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Separation and Purification
Section:
Summary of Activities
July 1970 to June 1971

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NATIONAL BUREAU OF STANDARDS

The National Bureau of Standards[1] was established by an act of Congress March 3, 1901. The Bureau's overall goal is to strengthen and advance the Nation's science and technology and facilitate their effective application for public benefit. To this end, the Bureau conducts research and provides: (1) a basis for the Nation's physical measurement system, (2) scientific and technological services for industry and government, (3) a technical basis for equity in trade, and (4) technical services to promote public safety. The Bureau consists of the Institute for Basic Standards, the Institute for Materials Research, the Institute for Applied Technology, the Center for Computer Sciences and Technology, and the Office for Information Programs.

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Office of Standard Reference Data—Office of Technical Information and Publications—Library—Office of Public Information—Office of International Relations.

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[1] Headquarters and Laboratories at Gaithersburg, Maryland, unless otherwise noted; mailing address Washington, D.C. 20234.
[2] Part of the Center for Radiation Research.
[3] Located at Boulder, Colorado 80302.

UNITED STATES DEPARTMENT OF COMMERCE
Maurice H. Stans, Secretary

U.S. NATIONAL BUREAU OF STANDARDS • Lewis M. Branscomb, Director

NBS TECHNICAL NOTE 589

ISSUED OCTOBER 1971

Separation and Purification Section:

Summary of Activities – July 1970 to June 1971

David H. Freeman and Walter L. Zielinski, Jr.
Editors

Separation and Purification Section
Analytical Chemistry Division
Institute for Materials Research
National Bureau of Standards
Washington, D.C.   20234

The Analytical Chemistry Division was established as a separate division at the National Bureau of Standards on September 1, 1963, and became part of the Institute for Materials Research in the February 1, 1964, reorganization. It consists at present of nine sections and about 100 technical personnel encompassing some 60 different analytical competences from activation analysis and atomic absorption to vacuum fusion and x-ray spectroscopy. These competences, and in turn the sections which they comprise, are charged with research at the forefront of analysis as well as awareness of the practical sample, be it standard reference material or service analysis. In addition it is their responsibility to inform others of their efforts.

Formal publication in scientific periodicals is a highly important output of our laboratories. In addition, however, it has been our experience that informal, annual summaries of progress describing efforts of the past year can be very valuable in disseminating information about our programs. A word is perhaps in order about the philosophy of these yearly progress reports. In any research program a large amount of information is obtained and techniques developed which never find their way into the literature. This includes the "negative results" which are so disappointing and unspectacular but which can often save others considerable work. Of importance also are the numerous small items which are often explored in a few days and which are not important enough to warrant publication-- yet can be of great interest and use to specialists in a given area. Finally, there are the experimental techniques and procedures, the designs and modifications of equipment, etc., which often require months to perfect and yet all too often must be covered in only a line or two of a journal article.

Thus our progress reports endeavor to present this information which we have struggled to obtain and which we

feel might be of some help to others. Certain areas which it appears will not be treated fully in regular publications are considered in some detail here. Other results which are being written up for publication in the journal literature are covered in a much more abbreviated form.

At the National Bureau of Standards, publications such as these fit logically into the category of a Technical Note. We plan to issue these summaries for all our sections. The following is the fifth annual report on progress of the Separation and Purification Section.

W. Wayne Meinke, Chief
Analytical Chemistry Division

PREFACE

This is the fifth annual progress report of the Separation and Purification Section. The guiding philosophy of the Section is to concentrate our efforts on applications and improvements of the separations field which are most important for the achievement of national and scientific goals. This past year started with major needs to expand and strengthen our capabilities in the practice and understanding of liquid chromatography. We devoted all available energy to this.

The growth of interest in liquid chromatography has come from the cooperative NBS-NIH program in clinical chemistry, of the need with the NBS program aimed at ecological monitoring known as Measures for Air Quality, and several other smaller projects in the area of organic analysis. The chromatography of bilirubin, which we did with some difficulty two years ago, taught the frustration of working with an unstable compound having low solubility properties. Our present responsibilities provide a strong stimulation to our search for a way to predict the conditions for successful chromatography. The advantages of this are obvious.

Apparatus development was the important starting point since we required considerable flexibility to change design configurations, whenever needed. We were also faced with the need for an estimated 10 or more liquid chromatographic units, but a budget that would not permit this unless we built the apparatus economically. We were thus forced to explore the potential of do-it-yourself instrumentation. Fortunately, the instrumentation problem with liquid chromatography is much simpler than that in gas chromatography. The details of our apparatus development work are described at length in this report--with the hope that it may stimulate those who would enjoy the pursuit of liquid chromatography, but who are impeded by the lack of design information.

The scientific understanding of the stationary phase is the focal point for the efficient use of liquid chromatography. There are many kinds of stationary phases, which makes this subject extremely versatile and powerful in the carrying out of efficient chemical separations. There are also many opportunities to go wrong by non-productive trails with improperly chosen materials. The conventional separating materials for the organic chemist are silica gel and alumina, two inorganic solids that are extremely difficult to predict, control or reproduce in performance. In an attempt to simplify the problem of organic separations we decided to develop our own stable of stationary phases. Our reasoning followed from the recognition that the powerful ability of ion exchange resins for inorganic separations can be traced specifically to the decision to locate a specific kind of functional group upon an otherwise inert but porous network. For organic compounds, such functionalization can be tied to those interactions which lead toward complex formation. This pathway has proved exciting, and progress was made in the development of "interactive gel networks" which are reported here in terms of both the synthesis and the preliminary understanding of the theory.

The use of brand names are only for the purpose of description and indicates that the equipment was used by this laboratory and was found to be satisfactory. This does not imply that these are the best or only available components for the job.

The preparation of this report was expertly assisted by Mrs. Janice Hurst, secretary to the Section, and this we gratefully acknowledge.

<u>Special Notice of Hazard</u> to Users of Chloromethyl Methyl Ether: see page 48.

David H. Freeman, Chief
Separation and Purification Section

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SEPARATION AND PURIFICATION SECTION: SUMMARY OF ACTIVITIES
JULY 1970 to JUNE 1971

Edited by David H. Freeman and
Walter L. Zielinski, Jr.

ABSTRACT

This is the annual progress report on the activities of
the Separation and Purification Section. During the past year
our studies in liquid chromatography, its application, control,
and systems design was continued at an accelerated rate.
Mobile phase flow was treated as an analogy to current in
direct current circuitry, following removal of liquid pulses
generated by a piston pump. A sophisticated, yet simple and
inexpensive liquid chromatograph design is fully described.
This design has been incorporated into all liquid chromatog-
raphy studies being conducted in this Section. The operating
parameters of this system design are discussed. The development
of the underlying theory and synthesis procedures for inter-
active gels has led to the control and prediction of solute
retention, selectivity, and a magnified column capacity for
use in fraction collection and ancillary characterization of
solute impurities in clinical Standard Reference Materials.
A gas chromatographic method is described for the analysis of
nitrilotriacetic acid based upon the formation of its trisilyl
ester derivative.

Key words: Clinical Standard Reference Materials; fraction
collecting; gas chromatography; interactive gels; liquid
chromatography design; nitrilotriacetic acid.

1. LIQUID CHROMATOGRAPHY

A. Description of Apparatus

For the past several years, an extensive effort has been undertaken in our Section tailored towards the development and assembly of simple liquid chromatographic systems. A portion of this effort was described in last year's report [1]. The requirements for such systems are that they be inexpensive, versatile, easy to assemble and troubleshoot, and that they conform to state-of-the-art performance in liquid chromatographic analysis. This document will detail the components which we have incorporated into a working system, and the use of a functioning system for chromatographic separations. Our work has essentially been directed toward the use of gel packings, although other materials are equally applicable. This section of this report will illustrate the various materials used for the construction of a simple liquid chromatograph. A major objective of the report is to encourage an increased world-wide participation in studies involving liquid chromatography at moderate pressures using simple apparatus of excellent quality.

1. Precolumn Components.

In order to assemble an apparatus for the best performance of liquid chromatography, certain system requirements must be met. It is the aim of this section of the report to explain the specific design employed by this laboratory in the construction of a liquid chromatograph. It is hoped that some of the problems encountered in our earlier systems may be avoided by anyone desiring to enter the field of liquid chromatography for the first time.

A rack or mounting frame of some fashion is required to support the column and other components of the system. From the operators view point it is desirable to have the components mounted in a manner which presents the least difficulty for injecting samples and making minor adjustments. The general

scheme of the liquid chromatograph is provided in Figure 1
and Table 1.

Figure 1. General scheme of the liquid chromatograph.

Table 1. Identity and sources for components
identified in Figure 2.

Code	Major Components	Source
A	Glas-Col heating mantle	1
B	Round bottom flask, S/T 24/40, 500 cm^3	1
C	Condenser, water-cooled, S/T 24/40	1
D	Controlled volume miniPump, #196-31	2
E	Stainless steel air tube, 6 mm o.d.	1
G	Connector tube, 3 mm o.d. stainless steel	1
H	Silica gel filter tube, 6 mm o.d. stainless steel	1
I	Silica gel resistor tube, 3 mm o.d. stainless steel	1
J	Pressure gauge, 0-300 lb/in^2, 5 lb/in^2 divisions	1
K	Cheminert sample injection valve, #R60SV, with ring stand mounting bracket, #R60MB	3
L	Sample loop	3
M	Luer syringe, glass	1
N	Sample vial	1
P	Precision bore column; 0.250" ± 0.0003" i.d., 1/2" nominal o.d., tooled on both ends 0.250" (tolerance of o.d. flange + 0.005", - 0.000")	4
Q	Liquid flow detector	5

Code	Fittings	Source
F1	400-2-2-316, Swagelok male elbow	6
F2	401-A-2-316, Swagelok male adapter tube to pipe	6
F3	400-3-316, Swagelok union tee	6
F4	400-C-316, Swagelok cap	6
F5	400-R-2-316, Swagelok reducing union	6
F6,7	200-6-316, Swagelok union	6
F8	200-4-316, Swagelok union cross	6
F9	100-R-2-316, Swagelok reducing union	6
F10	200-7-4-316, Swagelok female connector	6
F11	TEF107, Tube end fitting, and 107A3 coupling	3
F12	107B9, Female luer adapter	3
F13	TEF107, Tube end fitting	3
F14	687-053-0014, Stainless steel coupling with 688-358-0014 insert	4
F15	200-6-1-316, Swagelok reducing union	6
F16	100-6-316, Swagelok union	6

Table 1. Cont'd.

Sources

1	Standard sources
2	Milton Roy Company, P.O. Box 12169, St. Petersburg, Florida 33733
3	Chromatronix, Inc., 2743 Eighth Street, Berkeley, California 94710
4	Fischer & Porter Company, Warminster, Pennsylvania 18974
5	Laboratory Data Control, P. O. Box 10235, Riviera Beach, Florida 33404, and review article: J. Chem. Educ. 47, A549 (1970); ibid., 47, A675 (1970).
6	Crawford Fitting Company, 29500 Solon Road, Solon, Ohio 44139.

The first component of the liquid chromatograph proper is the reservoir (B) for maintaining a reserve of the mobile phase. In our work we have found that a round bottom flask of 500 ml or 1,000 ml capacity with a standard taper ground glass joint and a simple water cooled reflux condenser is adequate. The round bottom flask is placed in a "Glas-Col" heating mantle (A) and a conventional powerstat is used to control the current for sufficient heat to give a very gentle reflux (C) action. It is not advisable to boil the solvent, but rather to supply only enough to heat to drive off the dissolved gases in the liquid. From the reservoir a Teflon tube (T1) 1/8 in o.d. by 1/16 in i.d. is attached to a liquid metering pump (D) which is used to drive the mobile phase through the packed column. The two pumps most commonly used for this purpose are the reciprocating piston type and the high volume syringe type. Both types produce pressure pulses on each pumping stroke. The piston types normally operate at 15 to 35 strokes per minute and the syringe types operate at 1 to 4 strokes per hour depending on flow rates used.

For continuous operation the piston type pump is more desirable if the pressure pulses can be made negligible. A method has been devised by this laboratory to dampen the pulsation to a level that is not detectable by the liquid chromatographic monitors [2] in use. A flow resistive network

analogous to a simple electrical circuit is employed. Further details of this approach are covered in section 1.B.2.a. of this report. The construction and assembly details of the depulsing system are covered below.

The pump is used with our depulsing system, for driving mobile phases through columns at operating pressures of less than 500 lb/in^2.

An important element of the depulsing network consists of a 12 in length of 1/4 in o.d. standard wall stainless steel tubing (E). One end is capped so as to trap air in the tube and the other end is fitted to the pump with a tee (F3) perpendicular to the liquid flow stream. On the pumping stroke the air is compressed and on the filling stroke the air expands, analogous to the charging and discharging action of a capacitor. A resistive element (H) is placed in the line to increase the discharge time constant of the "capacitor," consisting of a length of 1/8 in o.d. standard wall stainless steel tubing packed with 30 to 60 μm particles of silica gel. Figure 2 is a
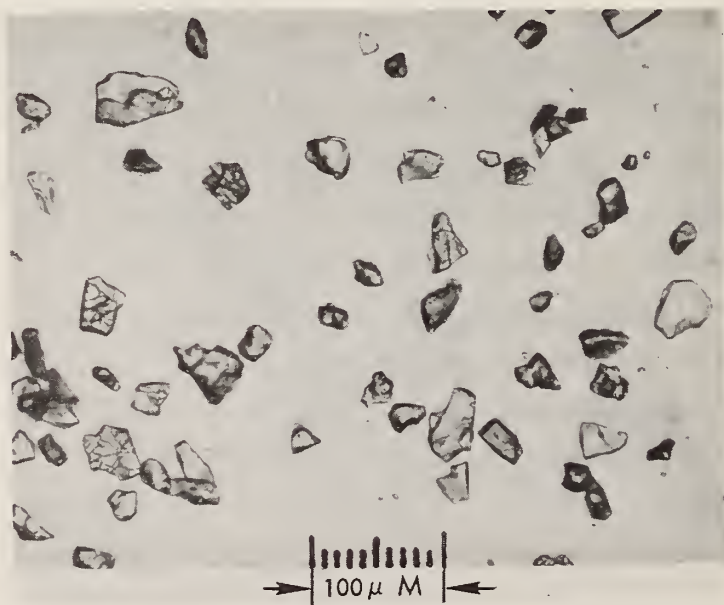
Figure 2. Photomicrograph of 30-60 μm silica gel used in resistance tubes.

photomicrograph of the silica gel with a 10 μm scale to show the size distribution of the particles.

2. The Reference Stream. The amount of resistance required in the reference stream (I) for balancing its effluent flow with that of the analytical column is determined by the equation given in section 1.B.2. of this report. The required length of steel tubing is filed flat and smooth across the ends and all burrs are removed. A 1/8 in diameter 400 mesh stainless steel screen disc is placed inside a 1/8 in stainless steel union. One of the connectors containing a screen is attached to the end of the tube and dry 30 to 60 μm silica gel is added. The silica gel is uniformly packed in the tube by tapping to a density of 0.52 g/cm^3 [2]. In order to verify proper packing of the tube it is advisable to weigh the resistor element before and after filling and calculate the packing density. Small scissors are used for cutting the wire gauze circles. The addition of silica gel to the tube is greatly facilitated by a small plastic wash bottle. Figure 3 shows the components used in construction of the resistor.

3. Pressure Monitoring. In order to monitor the stability of inlet pressure in the liquid chromatograph during operation a pressure gauge (J) is required at the head of the column. In our work we have found that expensive gauges or pressure transducers are not required for pressures of less than 500 lb/in^2. A simple bourdon tube gauge of the type normally used to monitor air or steam pressure when completely filled with the mobile phase has been found to be completely satisfactory for this purpose. Care must be taken to displace all air from the gauge by liquid; otherwise erroneous and erratic pressure readings will be obtained. The gauge is attached to the liquid flow line by a 2 to 3 ft length of 1/8 in o.d. Teflon tube to monitor the pressure at the head of the column. The gauge will also indicate system malfunctions as well as being useful to estimate flow through the system. A sudden rise in pressure

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Figure 3.   Components used in the construction of
resistor tube.

usually indicates a clogged line and a drop in pressure indi-
cates a leak in the system. A pressure versus flow plot is
useful for estimating flow rates and pump settings for a
particular experiment.

4.   The Analytical Column. The heart of the liquid
chromatography system is the column (P). It must consist of
an injection port (K, and Figure 4) for introducing samples,
it must be able to retain the packing in a stationary position,
and withstand the applied inlet pressures. The columns used
by our laboratory are commercially available precision bore
1/4 in i.d. heavy wall glass tubes with glass pipe flanges at
each end. Metal coupling units are used to secure a Teflon

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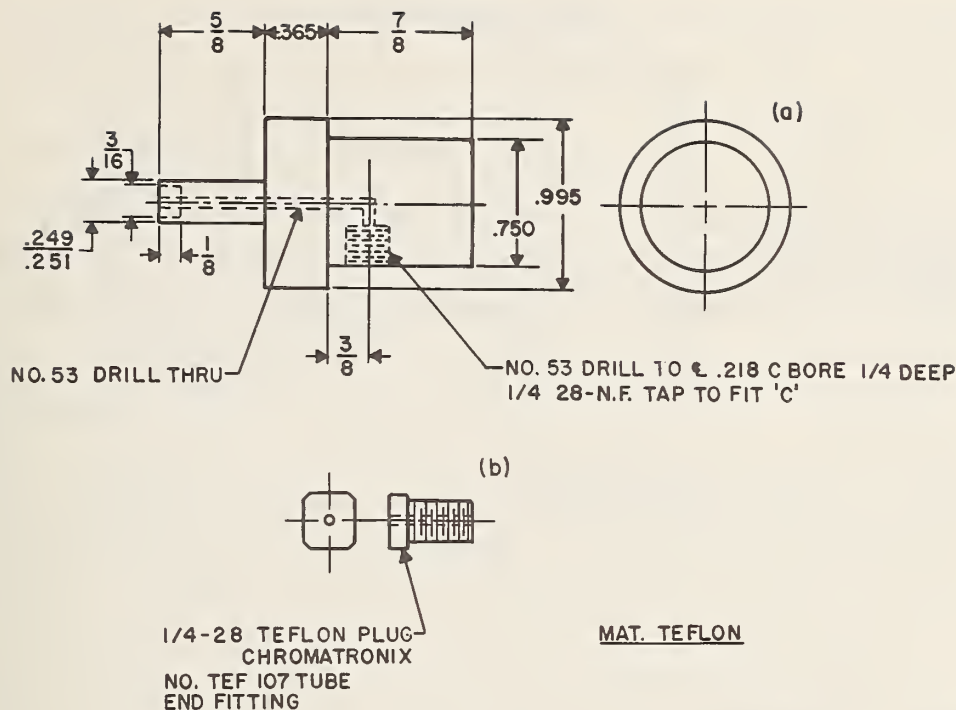
Figure 4. Column inlet fitting.

injection port at the head of the column (Figure 4) and a
Teflon fitting containing a porous retaining plug at the end
of the column (Figure 5). The porous Teflon plug (nominal 5 μm
pore with 55 percent voids) is used to retain the column packing
in the end fitting. Figure 6 shows the components used to
assemble a liquid chromatographic column. Both of these Teflon
fittings were designed in this laboratory and are adapted to
the existing precision bore columns. Figures 4 and 5 show
plans for machining these fittings from Teflon.

Glass columns of 1/4 in i.d. of lengths varying from 4 in
to 4 ft have been used in this laboratory at operating pressures
up to 500 lb/in^2 with no difficulty. The technique of packing
the column is described in section 1.B.1.

5. Detectors (Q). The detectors we have principally used
for liquid chromatography are differential refractive index and
the differential ultraviolet absorbance monitors. In order to

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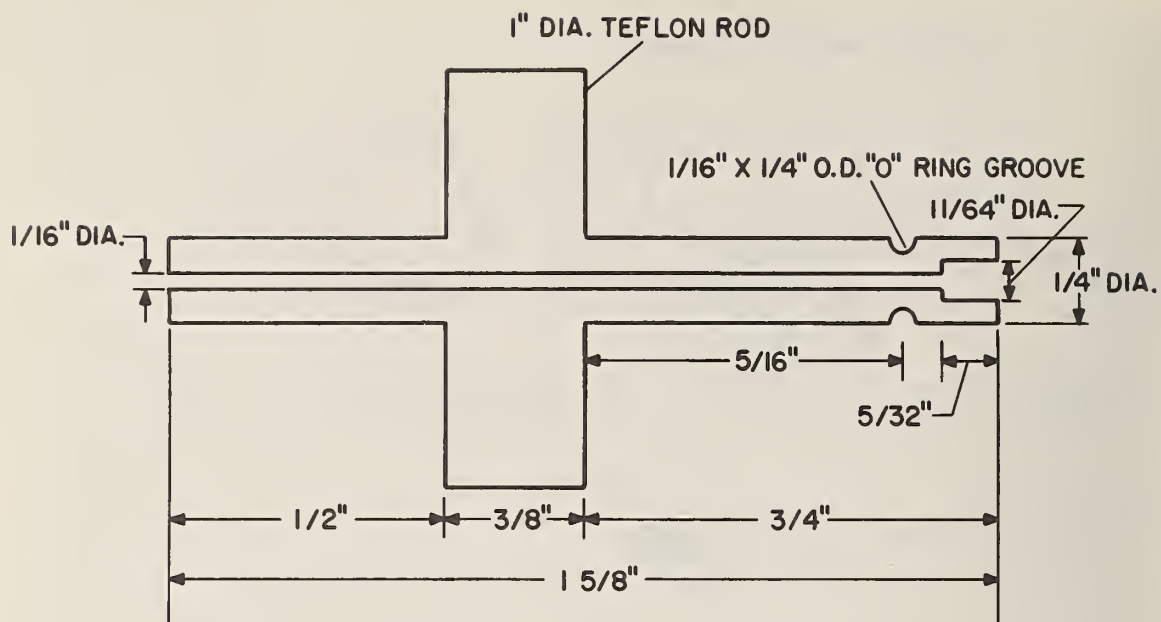
Figure 5. Column outlet fitting.

Figure 6. Column components prior to packing.

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obtain maximum stability and sensitivity from these detectors a
flow of the pure mobile phase is monitored as the reference
stream. The flow through both cells of the detector should be
similar. A direct method for obtaining equal flow in the two
streams would be to have two identical systems operating in
parallel, but this elaboration is not necessary. The analogy
of the liquid system to an electrical circuit is available
for the calculation of the resistance required to give balanced
flow through both the column and the reference streams (see
section 1.B.2.b.). In order to calculate the resistance needed
for the reference stream a flow rate measurement at a specific
column head pressure is required. After the value is obtained
a resistive element is made up as described above and installed
in the reference line. The detectors used for liquid chroma-
tography in this laboratory are the "RefractoMonitor" and the
"UV Monitor," Model 1250 (Laboratory Data Control). The
detector is always placed as near as is physically possible
to the end of the column. The column outlet fitting is
attached to the 1/16 in o.d. capillary tube leading to the
sample cell through a reducing union (F15). This technique
results in a low sample dead volume between column and sample
cell. Figure 7 is a close-up photograph of this column-to-
detector connection. In many instances it is desirable to
monitor a liquid chromatograph with the UV and the RI detectors
connected in series. The shortest possible length of 1/16 in
o.d. stainless steel capillary tubing should be used in
joining the detectors. In our work we obtain better results
when the RI detector is nearest the column. The UV detector
is less affected by the pressure drop generated by the
additional length of capillary tubing and it has a faster
response time because of the higher sensitivity. Both of
these detectors have a 25,000 ohm source impedence and are
connected to 10 mV potentiometric recorders to give a visual
display of the outputs.

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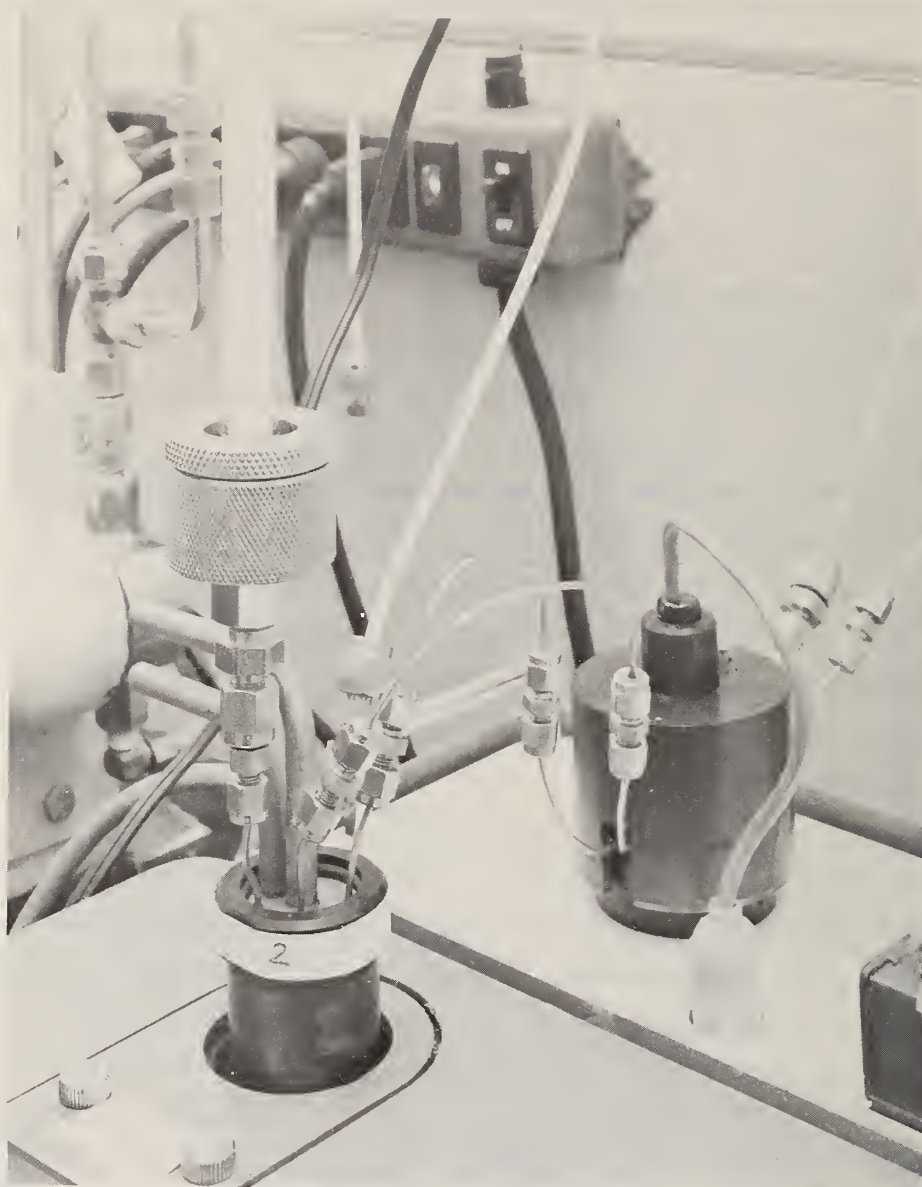
Figure 7.   Connection of column to detector.

6.   <u>Flow Measurement</u>.   In order to obtain precise effluent
flow measurements from the liquid chromatograph, an ordinary
10 ml or 25 ml capacity calibrated burette is modified by the
addition of a glass tube side arm just above the stopcock.
When a flow measurement is made the stopcock is closed and a
stop watch is used to time the flow between graduation marks

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of the burette. A calibrated 1 ml pipette has also been used for flow measurement in a connect-disconnect mode. Figure 8 shows a completely assembled liquid chromatograph as it is operated in this laboratory.
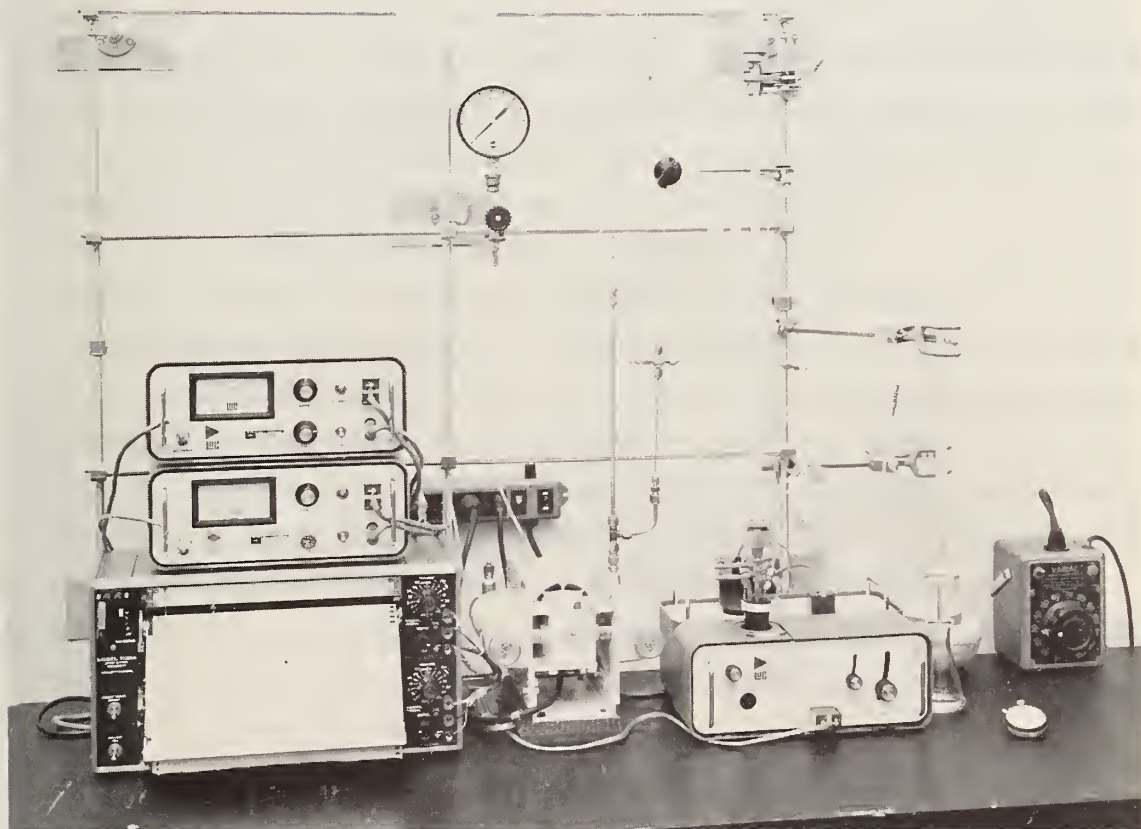
Figure 8. The assembled liquid chromatograph.

7. <u>Comments</u>. If high precision trace impurity analyses are to be achieved by liquid chromatography it is imperative that no contaminants be added by the system. In our efforts to achieve this objective, all systems are assembled such that only glass, Teflon, or stainless steel contact the stationary and mobile phases. The necessary interconnections between these three materials are provided by a variety of connectors. For steel tubes and some of the Teflon tubing, "Swagelok" type

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connectors are most useful. The main disadvantage to this connector is that its use with many solvents causes the metal to become quite dry and the threads will begin to gall after several changes. The presence of fine silica gel is also a contributing factor to thread galling. Such connectors should be used in the system where the least amount of change is expected, and only be changed when absolutely necessary.

For operating pressures of less than 500 lb/in^2 "Cheminert" fittings (Chromatronix, Inc.) have proven to be very useful. These fittings are very versatile with 1/8 in o.d. and 1/16 in o.d. Teflon tubing, and adapters are available for use with pipe, steel tubing, glass tubing, and hypodermic syringes. The difficulty with the use of this connector is its use of a rubber spring washer to make the seal. A synthetic rubber washer should be selected which will be least attacked by the mobile phase in order to avoid possible contamination of the system. A special tube flanging tool (Chromatronix, Inc.) is easily used for assembling these end fittings and is necessary for preparing proper lengths of tubing and making repairs.

The injection valve used by this laboratory and some of the problems related to sample introduction are discussed in section 1.B.5. of this report.

B.   Parameters of Operation

The parameters of operation determine the output quality of any analytical device. Herein are described the essential characteristics for packing a column, the prediction and control of mobile phase flow, the control of column performance with respect to band spreading, and the general methods employed in operation of the chromatograph and generation of suitable data.

1.   Packing the Analytical Column. Any packing material may have associated with it a quantity of fines or excessively large beads that should be removed before good performance can be obtained in a chromatographic column. These are best

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separated by sedimentation or levitation in a liquid of slightly lower density than the packing.

A decantation procedure is easily employed for removal of fines. The sample is placed in a beaker large enough to provide sufficient volume for the addition of liquid at least 10 times the volume of the beads. Liquid is added and vigorously stirred for a minute and allowed to stand undisturbed. The settling time determines the sizing; at least an hour is needed for good results. The fine material in the supernatant liquid is poured off, and the procedure is repeated five times or more depending upon the proportion of fines to be removed.

We find that packing a column with particles in the range of 10 to 30 microns results in good column efficiency.

Gels used as packings are usually also contaminated with residual reactants from chemical synthesis. The purification procedure depends upon the particular type of problem. One example is the use of soxhlet extraction. A simple washing procedure may suffice. The sample is placed in a flask with the solvent and stirred, or agitated ultrasonically, and filtered through a Teflon micro-filter. Additional washes can be used, with a final step of using the same solvent that will become the mobile phase.

Interactive gels (section 1.D.3.) which have been used for a long time with a variety of compounds in large sample sizes may become contaminated. It may be necessary to clean these gels with an acidic wash such as a solution of 5 percent acetic acid in chloroform followed by a basic wash of 5 percent triethylamine in chloroform if the gel is basic in character. The reverse order applies if the gel is acidic. In either case, the gel is finally washed with mobile phase solvent.

The column packing procedure for polymeric gels is as follows. The filtered gel is slurried in the mobile phase liquid. The slurry should be thick enough to flow as a uniform mass when poured. In preparing the slurry, dispersion of the beads is enhanced by ultrasonic agitation. This may be done

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in a small filtering flask, the side-arm of which serves as a convenient pouring spout in packing the column.

Before filling the glass column, it should be made clean: rinsing with acetone, followed by washing with a warm mild detergent solution, scrubbing the interior with a long brush. The column is rinsed with distilled water and allowed to dry thoroughly.

The bottom fitting containing a porous Teflon plug is placed in one end of the column, which is then clamped vertically. The packing slurry is poured as quickly as practical down the inside of the column, permitting air to escape from the top. This initial pouring should fill the entire column. The excess liquid is allowed to drain through the bottom fitting.

At the end of a few minutes the formation of the bed at the bottom of the column will become apparent. Bed formation will occur at a decreasing rate. The supernatant liquid is then removed by inserting an inert plastic tube into the top of the column, and aspirating the liquid into a filtering flask. It is extremely important to leave at least one half centimeter of liquid above the bed. If liquid level is lowered to the top of the bed, a zone will appear on subsequent addition of slurry, indicating non-uniform packing. This zone will form even if the top of the bed is stirred prior to adding slurry.

The procedure of removing supernatant liquid and adding slurry is continued as long as this is practical. The top fitting with its connections to pump and pressure gauge is then fixed in place, and pumping of the mobile phase is begun. Pressure is maintained well below that to be used in the chromatography. As pumping continues, the bed will settle and nearly clear supernatant liquid will appear at the top. As before, this is removed, fresh slurry is added, and the pumping is repeated until the column is completely packed.

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When the column is completely packed, the system is
connected and the pressure is raised to the operating rate
of liquid flow. It is desirable to pump liquid through the
column for a substantial period before putting it into
analytical operation. This can be done overnight. If further
compression of the bed occurs during this period, the addition
of a small amount of slurry will be necessary.

Chloroform is often used as a mobile phase solvent in
liquid chromatography using copolymer gels. Some difficulty
is encountered upon packing columns longer than about two feet.
The beads tend to float because of the greater density of the
chloroform. When packing is done with solvent flow in the
vertical downward direction, at first the bed will form and
even grow in length. This growth will cease when the condition
arises where the beads flow in an endless elliptical motion
without adding to the bed depth.

This difficulty can be overcome and the column success-
fully packed by preparing the slurry with a mixture of 2.5
parts of benzene to 1 part chloroform. The lesser density of
this mixture will allow the beads to settle. After the column
has been packed, the benzene is washed out by pumping chloro-
form through the column overnight.

2. <u>Mobile Phase</u>. The selection of the appropriate mobile
phase for liquid chromatography is governed by the analysis
desired, the column material employed, and to some extent, by
the detector being used. The mobile phase liquid and the
stationary phase packing must be compatible. If one is per-
forming standard steric exclusion (gel permeation) or inter-
active gel chromatography (see section 1.D.3.), it is a
requisite to use a mobile solvent which swells the gel,
allowing solute permeation to the gel interior to occur.
(In liquid-liquid chromatography, it is necessary that the
mobile and stationary phases have high relative inmiscibilities.
For brush particle columns, very polar alcohols such as methanol
will hydrolyze the interactive sites from the silica.)

In regard to detectors, the flame detector requires that at least a portion of the solvent adhere to the moving wire, which limits applications for aqueous mobile phases. In the UV detector using single wavelengths, it is imperative that the mobile solvent has low absorbtivity at the measuring wavelength to minimize energy losses in the incident light beams. The refractive index detector requires that the index of the mobile phase be dissimilar from that of the eluting solutes. Furthermore, the refractive index change of the mobile phase with change in ambient temperature ($\delta n/\delta T$) should be small, to avoid excessive baseline drift and cycling. This can be controlled by thermostating the detector cell.

In a properly designed liquid chromatographic system, the flow rate of liquid through the reference and analytical column streams should be both balanced and predictable. This aspect of system design is described below.

a. <u>Depulsing</u>. Liquid chromatographs operating under inlet pressures below 1,000 lb/in^2 commonly have mobile phase flow provided by piston pumps. Unless the pulsed mobile phase flow leaving this type of pump is damped sufficiently, the pulses will produce bothersome signal oscillations in high-sensitivity, low-volume detectors. The system we employ to minimize pulses to <1 percent at inlet pressures up to 300 lb/in^2, is shown in Figure 9. The system is analogous to an RC filter in an electrical circuit.

b. <u>Flow Balancing</u>. Flow balancing in the analytical column and reference streams is achieved by placing an appropriate resistor in the reference stream which matches the parallel resistance in the analytical column stream. The latter is achieved by simple analogy to DC circuits. From Ohm's Law, $R = \Delta P/I$, where ΔP is the pressure drop across the analytical column, and I, the effluent flow rate from the column. Combining Ohm's Law with D'Arcy's expression for laminar flow through circular porous conduits, it is possible
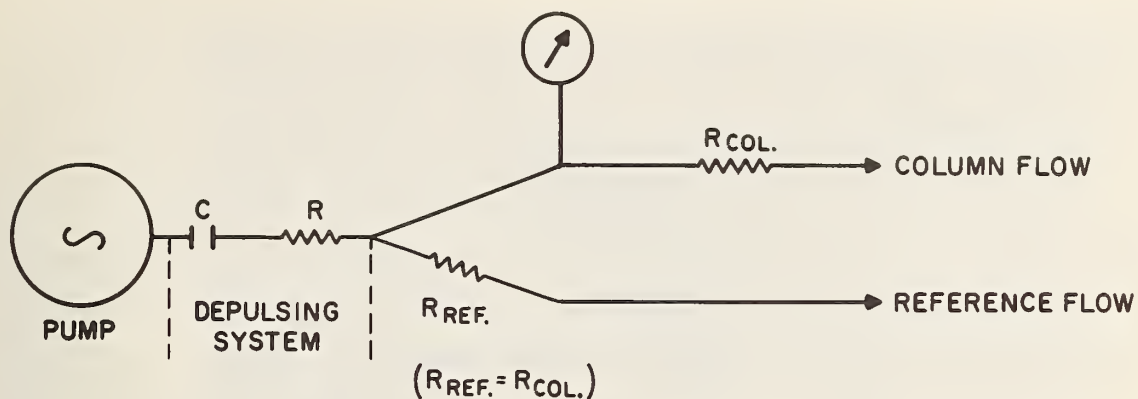
Figure 9. Schematic of depulsing and balanced
flow system.

to calculate the length of a resistor tube of given cross-
sectional area, packed with hard porous particles (eg. silica
gel; whose permeability (k) has been previously established),
which matches the resistance of the analytical column. For
3 mm o.d. x 1.6 mm i.d. stainless steel tubes, the length (L)
of a tube (in cm) packed with 0.52 g/cm^3 with 30 to 60 µm
silica gel (Figure 3) (k = 4.86 x 10^{-9}cm^2) necessary for flow
balancing, is given by

$$L = 8.5 \times 10^{-14} \, R_c/\eta$$

where R_c is the analytical column resistance in lb/in^2-min/cm^3
and η is the viscosity of the mobile phase. Once flow matching
has been established (Figures 9, 10) at a given inlet pressure,
it should remain so, independent of inlet pressure, provided
the analytical column resistance is a negligible function of
inlet pressure. Greater details of this work are given in
reference [2].

　　　3. Column Efficiency. An increase in column efficiency
is synonomous with a decrease in solute band spreading. Prior
to an investigation of column efficiency are the requirements
(1) to minimize the dead volume of all extra-column connections

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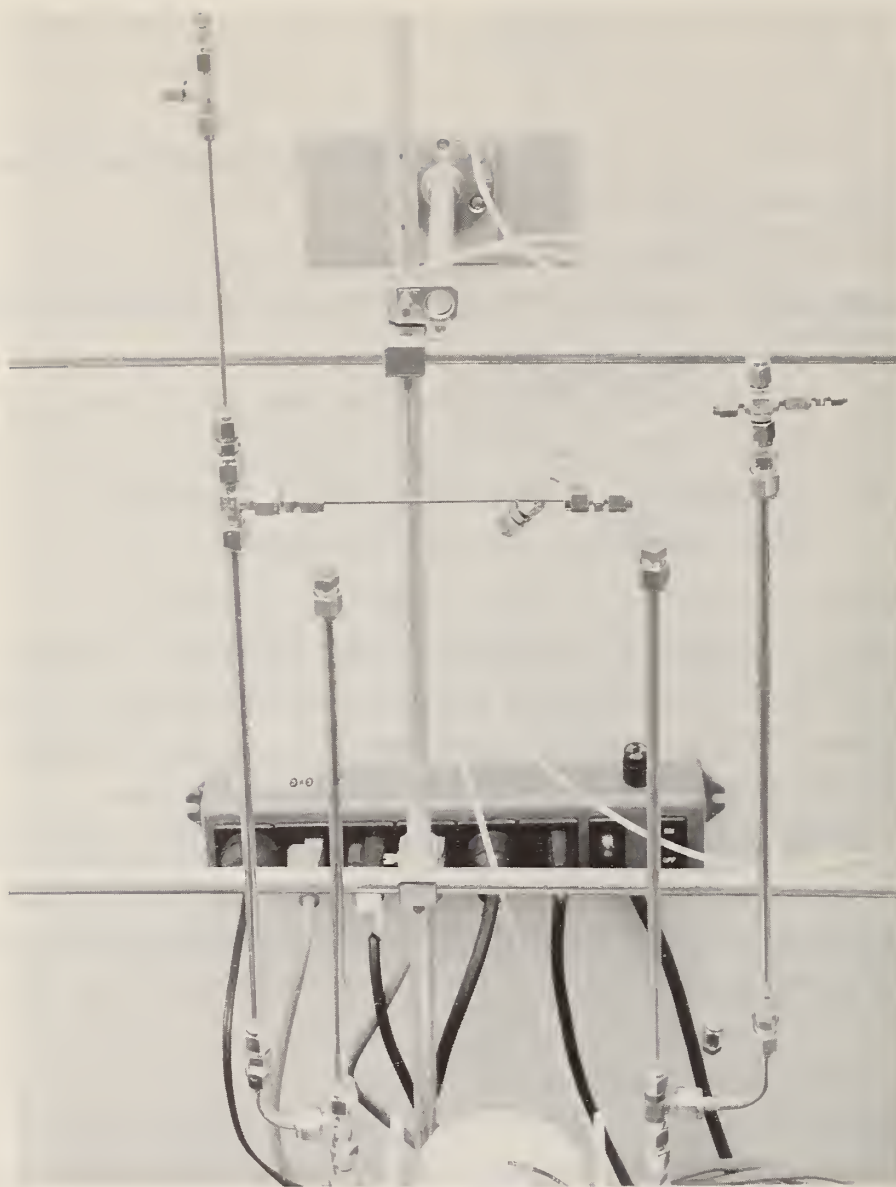
Figure 10.   Illustration of balanced flow system.

and plumbing and (2) to use small, concentrated samples of
solutes dissolved in the solvent used as mobile phase for sample
introduction into the chromatograph. An excess of external
plumbing volume does not contribute as greatly to solute band
spreading in liquid as in gas chromatography due to the 10^4
slower diffusion rate for liquids than for gaseous solutes.

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Minimization of extra column plumbing volumes is nonetheless, important in obtaining high system efficiencies. The use of small sample volumes is sometimes impossible when the solutes have low solubilities in the solvent used or when solute detectability by the detector being used, is low. Sample loops are especially effective for solute introduction, rather than syringe injection. When performed properly, this use of injection valves is more accurate and more reproducible than any other form of sample introduction.

The use of small uniform and spherical particles (eg., 10 to 30 μm) as column packing in liquid chromatography increases column efficiency. The use of small column diameters has some effect, but due to the slower diffusion of liquids is not as pronounced as in gas chromatography. Flow rate can be quite important, particularly for gel packings where permeative fractionation controls the separation [1]. In such cases, extremely slow flow rates (to 0.1 cm/min) produce the highest efficiencies. The construction of a van Deemter plot will quickly establish the best choice of flow rates to use for any kind of chromatography.

4. <u>Fraction Collecting</u>. The use of a fraction collector with a liquid chromatographic column is important in order to (1) establish solute stability during chromatography, (2) perform a mass balance analysis on separate components with reference to the measured mass of sample originally introduced, and (3) for subsequent determination of the separated constituents. Subsequent thin layer chromatography is especially powerful as a complementary analytical tool. Collection of fractions from liquid chromatography is easily achieved on a chosen time scale, with conventional collectors (Figure 11). Unfortunately, no commercial collector is available which permits variable time collection for fractions of the same analysis. Knowing the profile of the chromatogram it may be desirable to collect the entire valley between widely separated peaks into a single collection tube. Similarly,
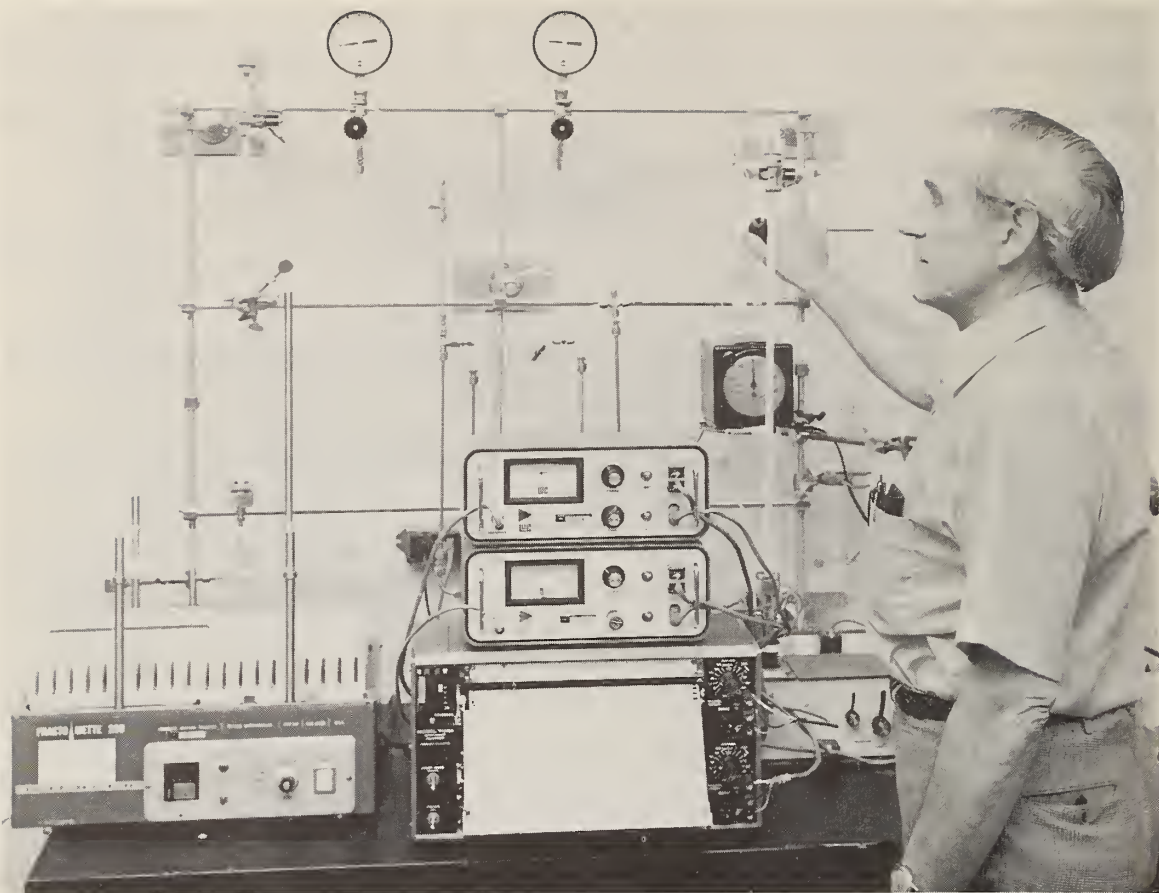
Figure 11. Liquid chromatograph including fraction
collector.

separated peaks can be collected into single tubes. This may
be achieved by storing appropriately placed signals in a com-
puter whose memory is swept at a fixed frequency. When a
signal channel is reached during the computer sweep, an actuator
circuit is tripped, advancing the collecting tube tray by one
tube location. The schematic of the actuator circuit designed
for coupling to our Fabri-Tek 1070 computer is shown in
Figure 12.

When only a very small amount of a sample is collected,
the appropriate fraction (or combined fractions) may require
concentration by solvent removal. A fraction concentration
system is shown in Figure 13. An example of the success of

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Figure 12. Schematic of actuator circuit for
fraction collector.

suitable sample concentration is given under the section of
Peak Authentication in Figure 14 which shows the IR spectrum
of 10 μg of cortisol using a micro-pellet and a beam condenser.

5. Sample Introduction: Methods and Problems. Injection
of the sample to be chromatographed is made with the system at
full operating pressure and stable conditions. Injection can
be performed in either of two ways: (1) by piercing a rubber
septum with a syringe needle, or (2) by means of a sample valve.
Each method has advantages and disadvantages.

Use of the needle and septum permits the injection of
small samples, 5 or 10 microliters, for example. Injections
may be made even at pressures of 200 lb/in^2 or higher especially
if a fixed-needle syringe is used. This type of syringe avoids
leakage at the luer fitting of demountable syringes which
routinely occurs at high pressures. At high pressures, pene-
tration of the septum results in small beads being driven into
a fixed needle so that not only is it impossible to inject the
sample but there also is the problem of a clogged needle

Figure 13.   Concentration of collected fractions.

resulting.   Removal of the particles is difficult, and may
necessitate discarding the syringe.

When syringes with replaceable needles are used, clogged
needles are a less serious problem.   With experience and
patience, a small wire can be poked down the barrel of the
needle to dislodge particles.   The needles can also be placed
into an ultrasonic bath which, with luck, may dislodge jammed
beads.

A serious disadvantage of the two element syringe compared
to the fixed needle type is the problem of leakage at the hub
of the needle.   This is particularly troublesome at pressures
of about 200 lbs/in² or more for most needles, depending upon

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Figure 14. Infrared spectrum of 10 µg of cortisol
obtained by concentration.

the fit of the needle at the luer. With samples of small size
this leakage may be unnoticed and time may be lost waiting for
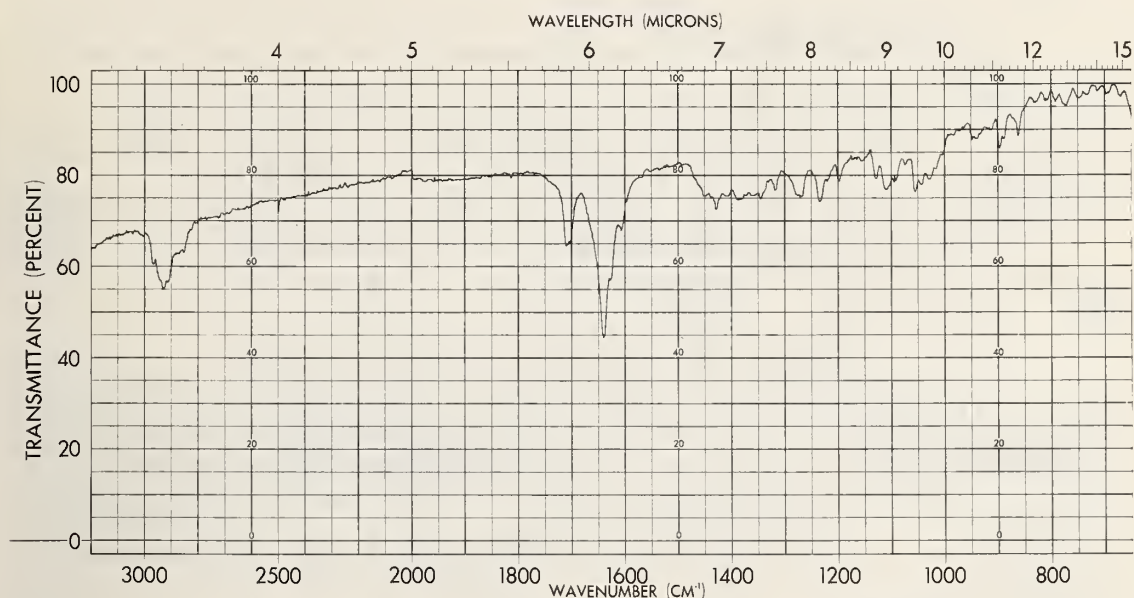the chromatography of a sample which, indeed, had never been
injected.

When larger concentrated samples need to be injected for
the collection of fractions, needle injections against even
moderate pressure is difficult due to the back pressure on the
relatively large plunger of larger sized needles.

Septums themselves also give rise to problems. Chloroform
and THF swell and cause deterioration of most materials from
which septums are made. We have been able, however, to get
long service from viton septums when used in the type of
injection fitting developed in our laboratories and illustrated
in Figure 15. With this fitting only a small area of the septum
is exposed to the solvent.

Physical stability of the septum is not the only consider-
ation in the use of septums for injections. The solvent may
extract components from the septum which may show up as peaks
in the chromatogram. Such artifacts often appear in fixed

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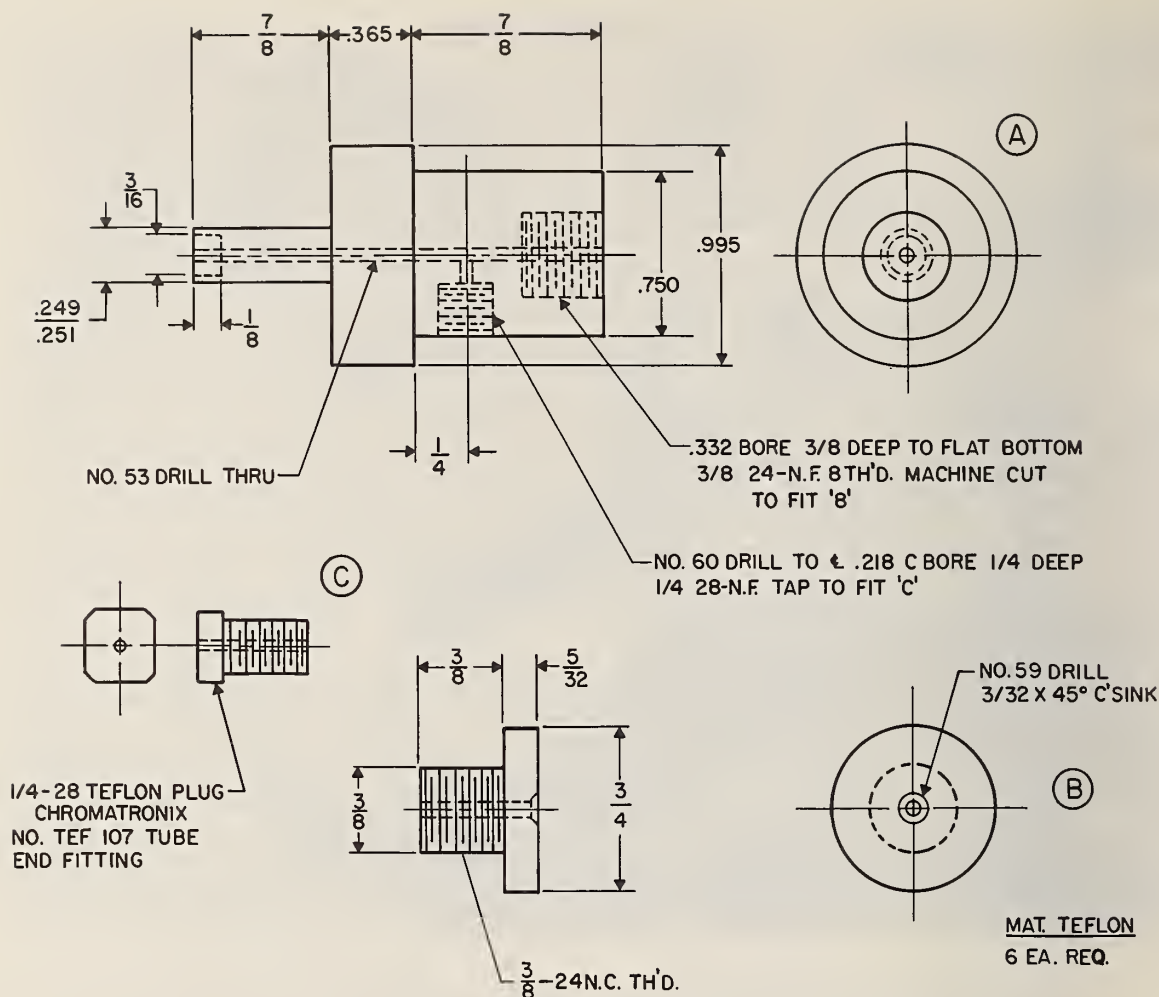
Figure 15.   Inlet fitting for syringe injection.

relation to the injection time indicating that the penetration
by the needle is a factor in the extraction of the septum
material. We should note here, another artifact which we
observed due to sample injection by needle through a septum.
In an earlier type of injection-fitting it was necessary to
use long thin needles in order to inject on the bed. It was
customary to direct the end of the needle into the small hole
in the injection port by the fingers of the left hand and indeed
to support it this way while pushing it through the septum and
into the column packing. Apparently, enough organic material

rubbed off the fingers onto the needle to show up in the chromatogram when a UV absorbance detector was used.

Injection valves of the type illustrated in Figure 16 have proven very satisfactory in our work. They are made of materials chemically inert to most solvents, and can be used with inlet pressures up to 500 lb/in^2.
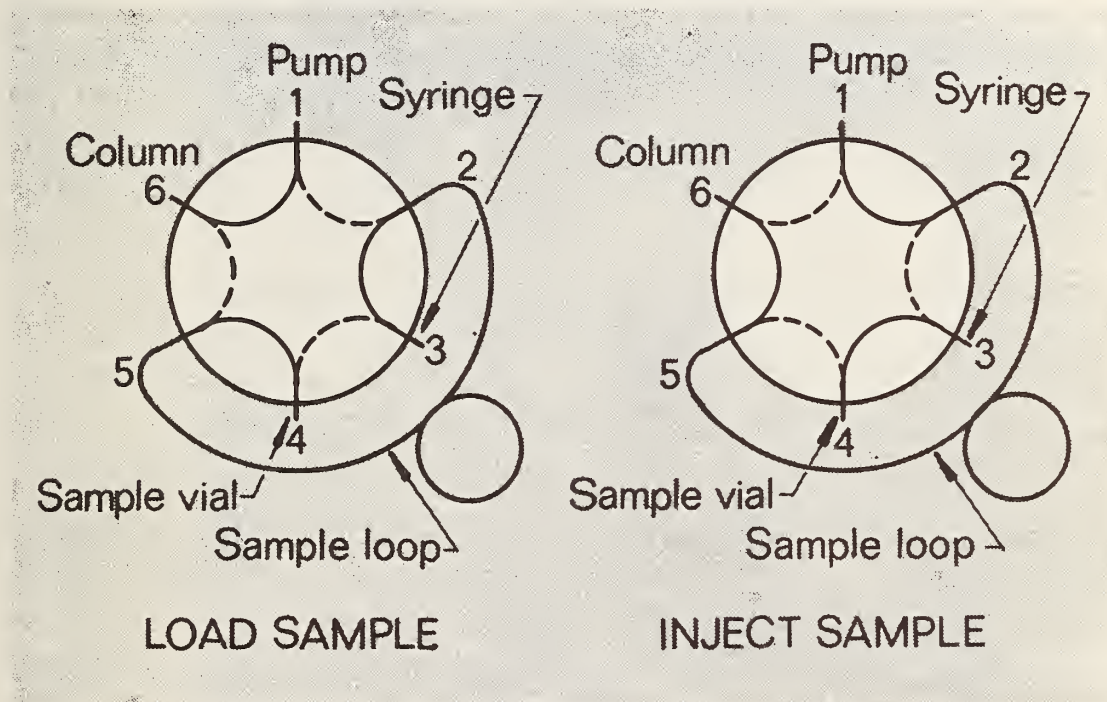
Figure 16. Two-way valve for sample introduction.

The operation of the valve is indicated in Figure 17. In the "load" position flow of solvent flows directly from the pump, through the valve, to the column. A sample is drawn from a vial into the sample loop by means of a syringe connected to port #3 by a female luer fitting. Turning the valve to the "inject" position switches the solvent flow from the pump, through the loop attached to the valve, delivering the sample into the column. Another sample may be injected at any time without stopping the solvent flow by repeating the procedure.

Figure 17. Sample introduction with the two-way valve.

Sample loops are easily interchanged, rinsed, or filled, without
admitting air into the system. The size of the loop has been
varied from a useful lower limit of about 50 microliters for
analytical work, up to 10 ml for collection of sample fractions.
It should be noted that the size of the injected sample is
larger than that of the loop by about 0.1 ml because of sample

dead volume contained in the connecting tubing and in one of the rotor passages.

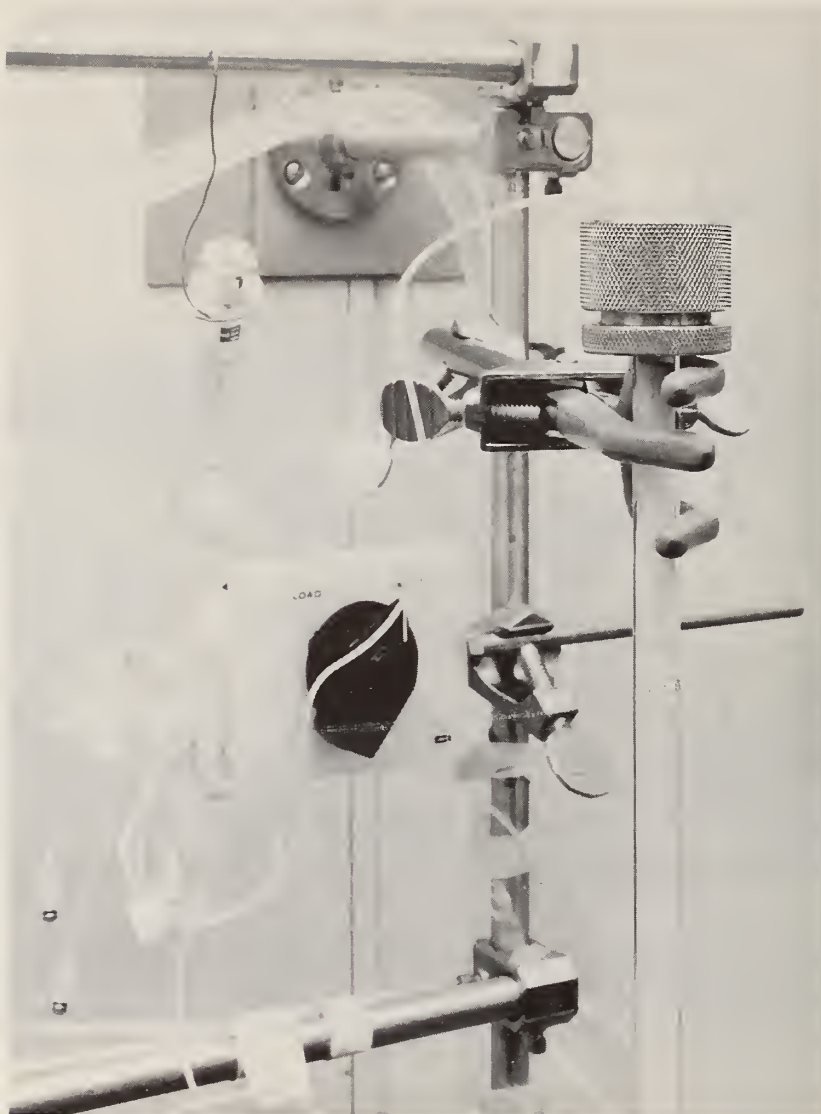It would seem that on-column injection, which is possible with needle and septum and not with valve injection, should result in less band-spreading. We have observed no significant difference in this regard.

Sample introduction valves of the kind discussed here are expensive, considerably more than a system composed of a needle and septum. However, when one considers the long-range cost due to attrition of needles and syringes, and even more importantly, the down-time arising from problems of septum leakage and malfunctioning of needles and syringes, the valve method is the preferred choice.

6. <u>Measurement of Retention Volumes</u>. A considerable portion of our work in liquid chromatography has involved precise measurement of solute retention volumes. The retention volume is simply the difference between the retention times of the solute and a reference solute, multiplied by the flow rate. For the purpose of flow measurement we have found convenient the use of a modified burette such as is represented in Figure 18. The glass side-arm is sealed in place and connected to the column exit port of the detector by plastic tubing. A luer-lock stainless steel connector permits uncoupling of the exit tubing for recycling the eluant to the solvent reservoir during stand-by periods such as overnight or overweekend.

To measure the flow rate, the stopcock is closed and the eluant flows up into the burette for a flow of 5 ml. During one or more periods of the chromatographic run, the time for a flow of 5 or more ml is measured with a stop watch. Flow rates measured in this way are usually reproducible with a precision of a few tenths of a percent which is also a reflection of the stability of the overall hydraulic system.

Day to day data, however, indicate a precision of 5 percent in retention volume measurements, although replicates of within-day values will agree to ± 0.1 to 0.2 cm^3. This may

Figure 18.   Flowmeter for measurement of effluent
             flow rates.

reflect changes that occur by compaction of the column packing.
For this reason, normalized retention volumes are used.   These
are obtained by reference of the solute retention volumes to
that of selected reference standards which are used each day,
either individually or as internal standards to the samples.

C.   Peak Authentication

The problems associated with the determination of the
identity of a peak may be magnified in proportion to the amount
of material required by the various analytical techniques
employed.   For example, while 10 µg can be used for IR,
less than 1 µg for UV (provided sufficiently high extinctions
exist for the component in question) and mass spectrometry,
1 to 10 µg for gas and thin-layer chromatography, NMR spectra
requires 100 to 500 µg of material.   The difficulty involved in
peak authentication is further complicated obviously, if com-
plete separation from other sample components is not attained
during liquid chromatography.   Further, to insure collection
of sufficient material of single sample components representing
less than 1 percent of the sample, large sample inputs (as high
as 100 mg or more) can result in some deterioration of
chromatographic resolution.   Approaches used in the various
techniques we have employed are described below.

1.   Spectrophotometric.   UV and visible absorption spectra
on collected liquid chromatographic fractions are most useful
in the favorable case where suitable standards for comparison
are available.   Qualitative IR spectrophotometry in contrast,
provides important information concerning the presence of
functional groups in the material.   Furthermore, absolute sample
identity has a greater degree of confidence from IR spectra
matching with the possible exception of closely related struc-
tures, than does UV and visible spectra matching.

The procedure we have used for obtaining IR absorbance
spectra from liquid chromatographic fractions involves sample
concentration by evaporating the solvent, making a KBr pellet
from the residue, and examining its infrared spectrum.   In
isolated instances it might be possible to identify an eluted
compound by examining it in the infrared while still in solution,
but routinely, the concentration of sample is too small and the
absorptivity of most solvents is too great.

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The technique of preparing a 13 mm KBr pellet from the residue remaining after evaporation of the solvent is generally restricted to samples having a total mass of 1 mg or more. As an alternate procedure, for samples in the range of 0.5 to 1 mg, we obtained better spectra by adding 150 to 200 mg of KBr powder directly to the solvent, prior to evaporation to dryness and preparation of a standard (13 mm) pellet. In both methods blanks were run representing a portion of column eluant collected during a segment of the chromatogram during which no sample components were eluting. Figure 13 is a picture of the rotary evaporator used for concentrating eluant fractions.

In the more difficult case where very small amounts of a compound is obtained from the chromatograph, a similar but more careful technique is employed. Care must be taken to insure that containers and reagents used in the preparation of the KBr pellet are scrupulously clean. This technique involves the addition of 1 mg of KBr powder to the tube containing the eluted peak. The solvent is then carefully evaporated and the KBr-sample residue is used to prepare a micro pellet (1 mm diameter) using a micro die assembly (Figure 19). The micro pellet is then placed in a 4X beam condenser in the spectrophotometer and the spectrum is obtained (Figure 20). A beam condenser is essential when 1 mm KBr pellets are employed. Using this method a satisfactory identification was made of 10 μg of cortisol (Figure 14).

2. <u>Gas Chromatography-Mass Spectrometry</u>. Chromatography of a solute on a gas chromatographic column provides only a single piece of information, viz., a retention time, or more preferably, a retention volume. Chromatographic retentions on two dissimilar columns (eg., one "polar" and one "non-polar"), provides a higher confidence level for qualitative analysis, provided sufficient material is available for two injections. A. J. P. Martin early pointed out that solute mobility is a function of the contribution of the various substructures composing the solute molecule. Employing a two-column concept

Figure 19.   Microdye assembly for preparation of
1 mm KBr pellets.

and differential retention index analysis, it is possible to
obtain information related to sample identity from gas chroma-
tography alone, provided that the general structure class (IR
spectrophotometry can assist here) to which the solute belongs
is known (eg. steriods, sugars, etc.), and further that tabular
retention index information has been prepared for compounds of
the solute class (including the candidate compound). The GC-
data acquisition system is shown in Figure 21. The point
remains that this approach is able to differentiate among a
limited number of solutes, and is not very suitable for solute
identification when the number of possible solutes is large.

Information concerning sample identity is greatly improved
when a mass spectrometer is coupled to the chromatograph. Mass
spectral data coupled with the availability of an infrared
spectra of the compound will often suffice for sample
identification.

Figure 20. Infrared beam condenser (4X) containing
a 1 mm KBr sample pellet.

The thermal conductivity detection system and pneumatic
valving arrangement (Figure 22) for GC/MS analysis is such that
the GC column effluent is vented to atmosphere until a sample
component begins to emerge. This is then fed into the spectrom-
eter without introducing contamination. This system provides
that the chromatography study, the solute retention volumes,
and their respective mass spectra may all be obtained on the
same instrument. Use of retention values and mass spectral
information can assist in the authentication of the compound
of interest. Following identification, a solution is made up
using the same solvent as was used for the liquid chromatography
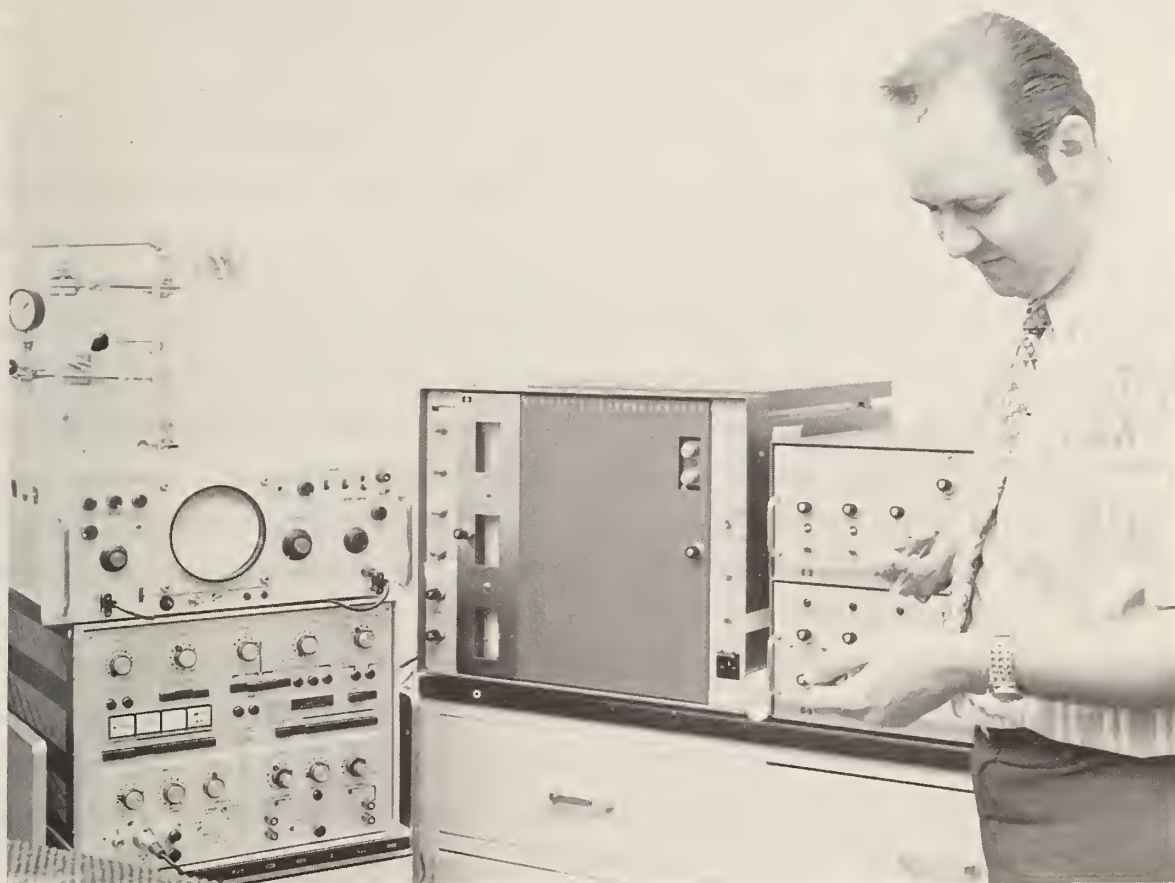and a small amount of the suspect compound from the shelf.

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Figure 21.   Gas chromatographic data acquisition
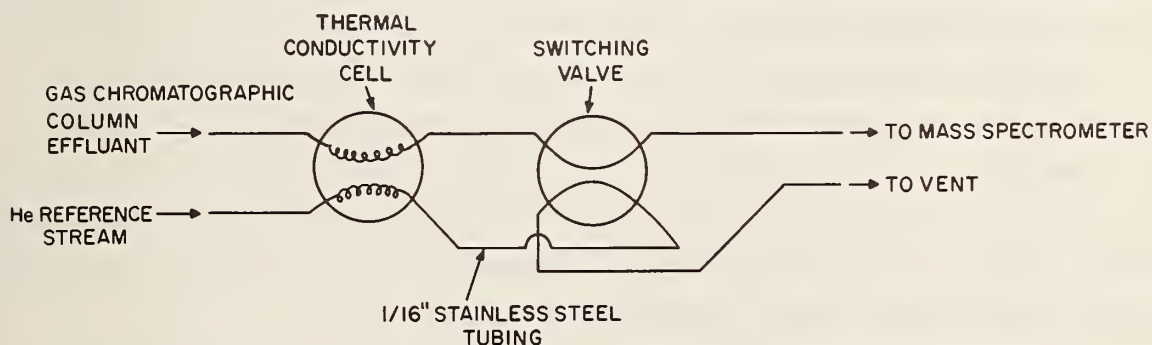system.

Figure 22.   Thermal conductivity detector and pneumatic
valving system for GC/MS analysis.

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This is analyzed in the GC under the same conditions; the retention times measured, and compared with the retention time of the unknown to double check the identification.

The detection/valving system within the GC is important from three aspects: (1) Our samples are frequently obtained in a large amount of solvent. By using this system, the solvent peak can be vented to atmosphere. This prevents a large solvent background in the spectrometer which might obscure the mass spectrum of the sample peak. (2) The ionization region of the mass spectrometer can become contaminated and this is also dependent upon the amount of sample put into it. By simple venting of solvent peaks, time between spectrometer shutdown and cleanout has been extended. (3) The thermal conductivity detector in the GC allows one to obtain a gas chromatogram each time a sample is run. If the MS is used both as a detector and peak identifier, the chromatogram resulting from total ion current signals are often inferior. The MS parameters for obtaining an acceptable chromatogram are slightly different from those used for producing an acceptable mass spectrum. When the MS signal parameters are changed during a chromatogram, spurious peaks were recorded, making the chromatogram unacceptable.

The mass spectrometer can be used in conjunction with the IR for non-volatile compounds also. If there is enough sample after solvent evaporation, a small quantity can be placed in a segment of a melting point tube sealed at one end and examined in the spectrometer using the solid probe sample entrance. This allows the sample, positioned at the tip of the metal probe, to be placed near the ionization region of the spectrometer. The "probe controls" allow external control of the sample temperature, up to 450 °C. Thus by careful heating of the probe to the appropriate temperature, the sample can be volatilized and a mass spectrum obtained.

3. <u>Proton Magnetic Resonance</u>. The approach taken here involves the collection of sufficient material from the liquid

chromatograph by applying high sample inputs. Good NMR spectra are attainable from 500 µg of collected material. We have in this manner been successful, in a cooperative effort with the Organic Chemistry Section, in obtaining the identity of a sample impurity present at less than one percent of the sample. This capability depends upon the special preparative features we have found with interactive gel chromatography.

4. <u>Computerized Measurement of Chromatographic Peak Areas</u>. A signal averaging computer was found useful for the electronic integration of chromatographic peaks. The electrical output of our gas chromatographs were available at amplificaions up to 15 volts. The interfacing was therefore no problem. For liquid chromatographic amplifications a simple 100-fold amplification was done using a bread board operational amplifier circuit.

In Figure 23 is shown a three component chromatogram with the integral superimposed. The integral is determined by taking the difference from the computer of digital voltage readings between the beginning of a peak where the slope of the integral is zero, and just after the peak where the slope changes from positive to zero.

D. <u>Column Packing Materials</u>

1. <u>Introduction</u>. Chromatographic separations occur because of the intrinsically different chemical properties of the components of a mixture, relative to the chemical properties of the stationary phase. These properties are transformed into corresponding elution velocities which determine the order and rate of component appearance at the column exit as the components travel through the column. The properties of the stationary phase in the chromatographic column is at the center of the action. Accordingly, stationary phase understanding is the key to successful liquid chromatography.

The selection of a successful stationary phase depends upon the criteria which indicate success. With modern high
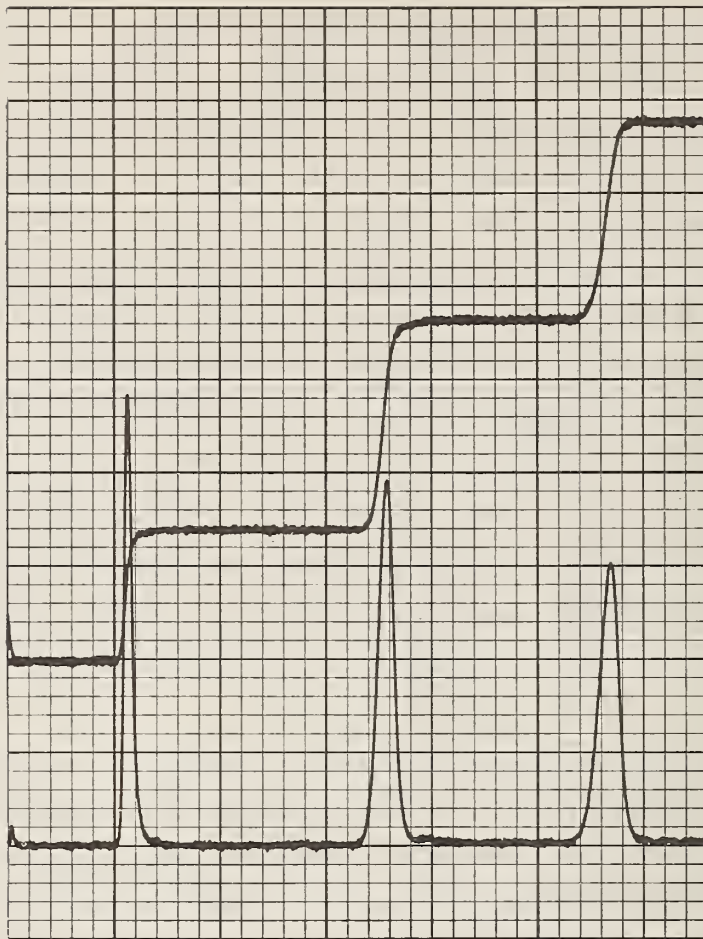
Figure 23.   XY recording of a chromatographed sample
showing peak integrals.

speed liquid chromatography it is a simple matter to demonstrate
a one-second separation of benzene and benzoic acid, although
the problem may be trivial. The solving of new problems is
approached in parallel reasoning to known solved problems,
or on the basis of intuition and experimental guesswork. The
rate of problem solving is clearly not a constant for all
scientists, nor for all problems.

There are six major factors which need to be put into
perspective in a discussion of stationary phase selection:
The physical properties determine mechanical performance: the
average particle size and its distribution, shape, porosity,

density, thermal stability, etc. The capacity determines the maximum sample size that can be handled per unit cross-sectional area of column; with surface adsorbants the capacity tends to be small, and with gels it is generally much larger. The available selectivity refers to the intrinsic ability of the stationary phase to interact differently with different solutes, and thus provide a reason to expect, at least in qualitative terms, that separation should or must occur. The predictability of solute elution volumes refers to the degree of accuracy involved in achieving in practice a precalculated elution volume for each solute component. The mobile phase moderateability is a concept which we use to indicate the benefit provided by options of solvent composition or effect of temperature in order to suppress excess solute affinity for the stationary phase. The available efficiency is properly described in terms of the number of theoretical plates per meter for a given column geometry, flow rate and temperature; it is the composite result of all chemical and physical factors that tend to diffuse the chromatographic peaks and thus impede the achievement of good resolution.

Table 2 shows a comparison of how we view the relative strengths of the different stationary phases. This is partly opinion. It should be noted that the availability of "high speed" characteristics is omitted since our view of speed includes the rate of problem solving, and this is adequately handled by the predictability of chemical performance.

2. Steric Exclusion Chromatography. The range of separations based upon steric exclusion (gel permeation) principles alone are principally dicated by the porosity of the gel and by the mobile solvent controlling the degree of gel swelling. The upper analytical (solute exclusion) limit is dictated by the largest solute structure which is just totally excluded from the gel interior, and this represents the elution volume of a "non-permeating" solute. The lower limit for analytical separations is based by the largest

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Table 2. Comparison of stationary phase types used in different forms of liquid chromatography.

	Selectivity	Predictability	Reproducibility	Efficiency	Capacity	Moderateability
Liquid/Liquid Chromatography	*		*	*		
Gel Permeation Chromatography			*		*	*
Liquid/Solid Chromatography	*		*	*		*
Ion Exchange Gel Chromatography	*		*	*	*	*
Interactive Gel Chromatography	*	*	*	*	*	*

solute structure which can permeate essentially all interior corridors of the gel phase. Solutes larger than the former are totally excluded and have the same minimum retention volume; solutes smaller than the latter type exhibit total gel permeation and have the same maximum retention volume. Solute sizes in between these boundary limits may be separated from one another provided there is a sufficient difference in their effective molecular volumes (ie. in their relative degree of permeation). This intermediate region is defined as the analytical or working region of the gel in regard to sterically controlled separations.

The working region of the gel can be extended on the upper end by increasing the average pore diameter of the gel. This can be achieved by lowering the degree of crosslinking during

copolymer synthesis, or by choice of an appropriate polymerization solvent and synthesis conditions in which large "pores" are introduced during gel synthesis.

a. Measurement of crosslinking. Two separate studies with copoly(styrene/DVB) conducted in our Section (1) have revealed the location of adsorption bands in the infrared for para- and meta-divinylbenzene crosslinking, and (2) have shown that measurement of the degree of each type of crosslinking is feasible from IR absorbance measurements on both standard, and commercial (containing meta- and para-ethylvinylbenzene) styrene/DVB copolymers [3,4].

b. Performance. The use of steric exclusion chromatography systems for the separation of organic solutes has been described in detail in last year's report [1]. One finding which is worth repeating here is that the plate height of a partially permeating solute varies oppositely with the flow rate. Thus, solute separations by steric exclusion chromatography are improved under slow flow rate conditions.

c. Limitations. There are important limitations to steric exclusion chromatography. There is its intrinsically poor selectivity that leads to lengthy analysis times for multicomponent samples. This is required due to the narrow analytical elution volume range available for separations. In principle, such separations operate only on the basis of solute size discrimination.

3. Interactive Gels

a. Description. The introduction of an interactive functional group into a high porosity gel replaces the phenomenon of separation by solute size with the phenomenon of separations based upon solute-gel interaction. The use of interactive gels for the separation of solutes having zero permeation differences greatly extends the working regions for separations on a gel system.

An introduction to interactive gels, the rationale for their use, and their potential for analytical separations in

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liquid chromatography is covered in the reprinted article shown in Figure 24, which was recently published [5].

b. <u>Synthesis</u>. Various methods are available for synthesizing interactive gels for liquid chromatography. One consists of suspension copolymerization with at least one of the monomers containing a specified interactive functional group. Another method consists of derivatization of a copolymer to introduce specific functional groups into the network. In order to insure homogeneity, the copolymer beads are swollen in an organic solvent. The swollen state needs to be maintained throughout the synthetic conversion. In last year's report we described the preparation of nitrated styrene-divinylbenzene copolymer [1].

(1) <u>Acidic Gels</u>. A weakly acidic functional group, namely, $-CH_2OH$, has been introduced into the copolymer matrix by a two-step reaction. The method consisted of chloro-methylation of the copolymer beads swollen in chloroethyl methyl ether, followed by alkaline hydrolysis of the chloromethylated gel. The material is being evaluated in our studies on chromato-graphic separation of proton-accepting organic compounds. Figures 25 and 26 show the set-ups used for chloromethylation and alkaline hydrolysis, respectively. Another chromatographic material was obtained by conversion of a hydrogen-form sulfonic cation-exchange resin into the Ag^+ form. On the basis of the ability of silver salts, such as $AgNO_3$ and $AgClO_4$, to form charge-transfer complexes with benzene and polynuclear compounds [6], the material is being examined in our investigations on polyaromatic compounds that occur in air pollution.

(2) <u>Basic Gels</u>. A proton accepting network, a crosslinked polyvinylpyridine, was prepared and found to be effective for the chromatography of weakly and moderately acidic organic compounds. This material, copoly(2-methyl-5-vinylpyridine/0.08 divinylbenzene), is now commercially avail-able from Bio-Rad Laboratories. An infrared spectrum and a

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Interactive Gel Networks for Organic Separations

We wish to report a new and systematic basis for approaching the chemical separations of organic compounds. Interactive gel chromatography with a moving liquid phase is defined by three major factors: solvent absorption (imbibition), steric exclusion, and the combined chemical interactions among gel, solvent and solute. The third involves the reversible process:

$$\text{Acceptor} + \text{Donor} \rightleftharpoons \text{Complex} \qquad (1)$$
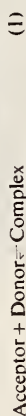
This can involve localized electron pair donation as in Lewis basicity, hydrogen bonds, or the more diffuse delocalized nature of pi electrons in unsaturated systems. Our preliminary findings with three different gel network structures are described in this report.

The experimental arrangement, in brief, involves the pumping of organic solvent into a 0.6 by 58 cm column containing the gel network in bead form (bead diameters of 10–30 μm). The effluent stream was monitored using low dead volume spectrophotometric (UV) and refractometric detectors[1] obtained from Laboratory Data Control. Under typical conditions an inlet pressure of 50 p.s.i. and a flow rate of 0.5 ml. minute^{-1} produced column efficiencies in the range of 200–600 theoretical plates for 5 mg. or less, of solute. The solute elution volumes (V_e, expressed in ml.) were measured with a relative standard deviation of approximately 5%.

The first gel was prepared using suspension polymerization[2] to obtain poly(2-methyl-5-vinylpyridine/0.08 divinylbenzene), or PVP. The swollen gel imbibed 65% by weight of chloroform solvent, used as the moving phase. The elution volumes for PVP with chloroform are given in Table 1. The sequence is: polystyrene which is sterically excluded, followed by the basic amines and the cyclohexane; these solutes show no obvious affinity for the PVP gel. A larger retention volume with aniline suggests the effect of the two slightly acid hydrogens. Then the alcohols emerge in order of their increasing acidity or degree of hydrogen bonding toward the pyridine nitrogen.

Table 1 Eluton Measurements on PVP Gel in CHCl$_3$

Solute	V_e (ml.)	log k	σ^*
Polystyrene †	6.95		
Et$_3$N	9.3		
Pyridine	9.8		
Cyclohexane	10.4		
Aniline	13.8		
t BuOH	15.6	−0.31	−0.30
i PrOH	19.0	−0.09	−0.19
n BuOH	22.0	0.03	−0.13
EtOH	26.2	0.17	−0.10
MeOH	42.2	0.47	0.0

† NBS Standard Reference Material No. 705.

The second gel was made of poly(styrene/0.12 divinylbenzene), or PSDVB, commonly used as a noninteractive stationary phase for steric exclusion ("gel permeation") chromatography. We based a prediction of chemical selectivity on the well known I_2: aryl complexation[3]. Chloroform was used as the moving phase with 61% chloroform imbibition by the gel. We found the following elution volumes: polystyrene, 6.3; benzene, 11.6; and iodine, 21.0. Again the polystyrene is sterically excluded, and benzene emerges later because of its higher permeation but otherwise essentially noninteractive behaviour in the gel. The obviously interactive behaviour and the moving colour of the iodine band are both striking.

The third gel was prepared as a dinitrated sample of the previous PSDVB material. Chloroform was used again as the moving phase and retention volumes for polystyrene of 6.8 and benzene of 9.1 were obtained. The values for pyridine, aniline and acetic acid, however, were all between 14 and 15. This gel seems to be amphoteric and the interactive processes seem to be levelled. The steric effects[4] remain.

The following will demonstrate how the complexation process, described in equation (1), can be related to the

Reference: Nature, Physical Science, Vol. 230, 135–36 (1971).

Figure 24. Interactive gel networks for organic separations.

chromatographic measurements. The ideal case (assuming an inert solvent, neglected activity coefficients, and 1 : 1 complexation) will be considered. First, the partitioning of the uncomplexed solute L ($\bar{L}$ in the gel phase) is described by

$$L \rightleftharpoons \bar{L}, \qquad D_0 = \frac{M_{\bar{L}}}{M_L} \qquad (2)$$

where D_0 is the molar concentration ratio for the uncomplexed solute species in the two phase (gel, solvent) system. The equilibrium complexation process is confined, by assumption, to the gel phase, and $\bar{G}$ is identified as the interacting moiety in the gel network

$$\bar{L} + \bar{G} \rightleftharpoons \overline{LG}, \qquad k = \frac{M_{\overline{LG}}}{M_{\bar{L}} M_{\bar{G}}} \qquad (3)$$

As in equation (1), $\overline{LG}$ is the acceptor–donor, or donor–acceptor, complex. The measured distribution coefficient is the combined ratio

$$D = \frac{M_{\bar{L}} + M_{\overline{LG}}}{M_L} \qquad (4)$$

Now we assume that D_0 is independent of the ability of the solute to interact, and that D_0 can be referred for measurement to an inert, but equally permeative, species. Then equations (2)–(4) are combined to give

$$k = \frac{1}{M_{\bar{G}}} \left(\frac{D - D_0}{D_0} \right) \qquad (5)$$

In the limit at vanishing M_L, the value of $M_{\bar{G}}$ becomes $M_{\bar{G}}^0$ and is obtained as the molar concentration of the functional moiety in the gel.

To illustrate, $M_{\bar{G}}^0 = 4.6$ was determined for the average aromatic ring in the PSDVB gel, and the value $k = 0.39$ l. mol⁻¹ is thus obtained for the iodine : aryl complex. This is a reasonable value by comparison with a predicted value of 0.20 for a comparable mixture of iodine with toluene and xylene in carbon tetrachloride, using the measurements of Andrews and Keefer[5].

In a similar way we have estimated the complexation constants for the interaction of the several alcohols with the PVP gel. These constants are obtained relative to cyclohexane (assigned D_0) and given as their logarithm in Table 1. The reader can see that our resulting log k correlates closely with the corresponding Taft σ^* value[6]. Thus the chromatography follows the inductive order for the alcohols: the lower the electron density on the oxygen the higher the relative complexation with the network pyridine group and the longer the relative retention volume.

We conclude that complexative interactions in a gel network are essentially identical to those which occur in simple liquid media. This implies the finding of a new and exact basis for predicting and interpreting the chromatography of organic molecules.

W. L. Zielinski, P. Byrne and H. Dixon of our laboratory helped with the development of the apparatus (work to be published).

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[1] Veening, H., J. Chem. Educ., 47, A549 (1970); ibid., 47, A675 (1970).
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Figure 25. Chloromethylation of copoly(styrene/DVB)
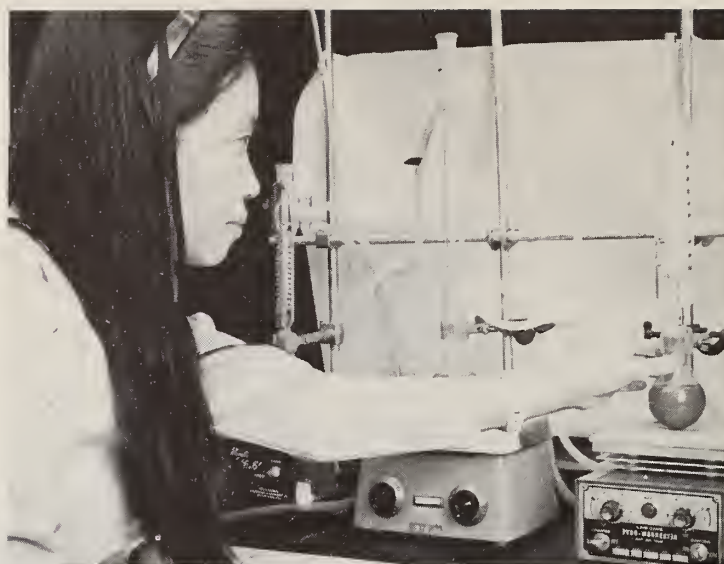   beads.

Figure 26.   Alkaline hydrolysis of chloromethylated
             copoly(styrene/DVB) beads.

photomicrograph of these copolymer beads are shown in Figures
27 and 28, respectively. We are now conducting experiments in
our laboratory on the synthesis of tertiary amine gels by
reduction of quaternary ammonium anion-exchange resins. This
synthesis is based upon the reaction of $LiAlH_4$ with quaternary
ammonium salts [7] as shown below.

$$RN^+(CH_3)_3I^- \quad \xrightarrow{\frac{LiAlH_4}{THF}} \quad RN(CH_3)_2 + CH_4$$
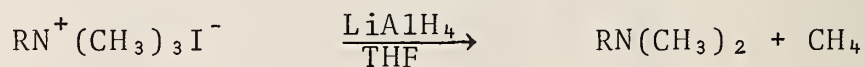
Other methods of converting anion-exchange resins into a
tertiary amine derivative based upon the Stevens and Sommelet-
Hauser rearrangements [8] are being studied. In the coming
year, gel modification by introduction of specific functional
groups into macroporous copolymer beads will also be
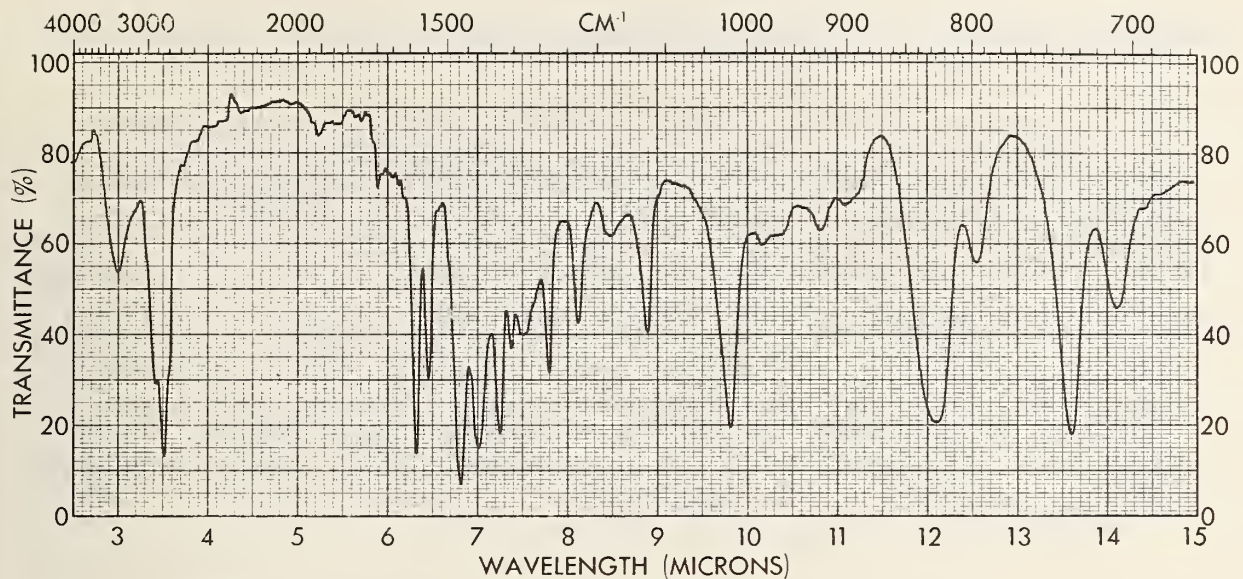investigated.

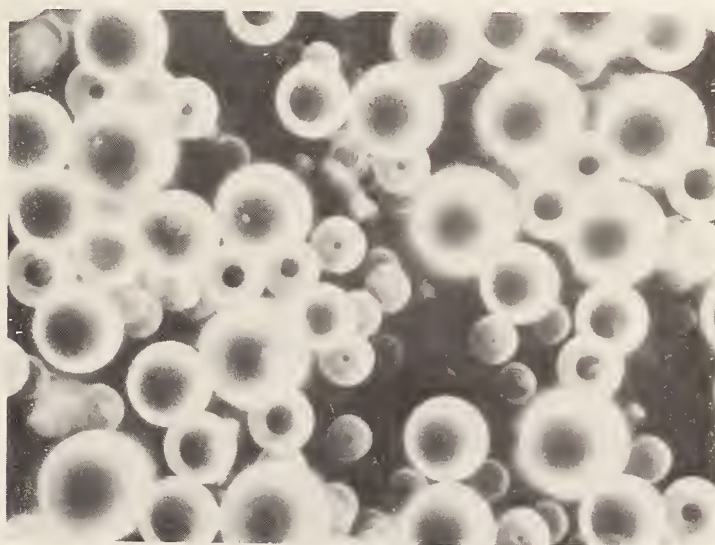Figure 27. Infrared spectrum of copoly(2-methyl-5-vinylpyridine/0.08 DVB).

Figure 28. Photomicrograph of copoly(2-methyl-5-vinylpyridine/0.08 DVB).

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Special Notice of Hazard to Users of Chloromethyl Methyl Ether

Chloromethyl methyl ether (CMME) may typically contain 1 to 10 percent (w/w) of an extremely dangerous carcinogenic impurity: bis chloromethyl ether (BCME). Results published by S. Laskin and coworkers [9] suggest that BCME may be among the most dangerous of all known chemicals. This is certainly true for the rat. Regardless, all users of CMME which may be contaminated by BCME are warned to adopt totally effective safeguards, to prevent inhalation or any other form of contact with the liquid or with its vapors.

2. GAS CHROMATOGRAPHY

A. Quantitative Analysis

1. Eicosane. Samples of >99.9 percent pure n-eicosane crystals, before and after high intensity gamma irradiation, were obtained for analysis. The work was performed on 12 percent (w/w) W98 silicone on 80/100 mesh Chromosorb W, AW/DMCS, using programmed temperature conditions. It was possible to detect n-alkane impurities below 0.01 weight percent. The crystalline n-eicosane was found to be unchanged by the irradiation, at least to within the tolerance of the measurements (0.1 percent).

2. Nitrilotriacetic Acid (NTA). This section participated with others during the past year in the development of methods for the measurement of NTA in water. The analysis involves two parts to the chromatography. Anion exchange chromatography is used first to free the NTA from complexed metal ions. We have concentrated our efforts on the measurement of the uncomplexed NTA by gas chromatography.

Since NTA itself $[N(CH_2COOH)_3]$ is nonvolatile, a gas chromatographic procedure would be impractical unless the NTA is derivatized to a volatile compound. NTA can be simply esterified using the silylating reagent "BSA"

$$
\begin{array}{c}
O-Si(CH_3)_3 \\
| \\
H_3CC{=}NSi(CH_3)_3
\end{array}
$$
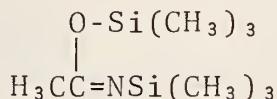
to form the volatile tri(trimethylsilyl) derivative [9]. This derivative was examined by GC using conditions similar to those described in this reference. The peak assigned to the tri-esterified NTA was verified using a mass spectrometer as the GC detector. The mass spectrum is given in Figure 29. To use the esterification procedure the NTA must be dry and in the protonated (3H) form.
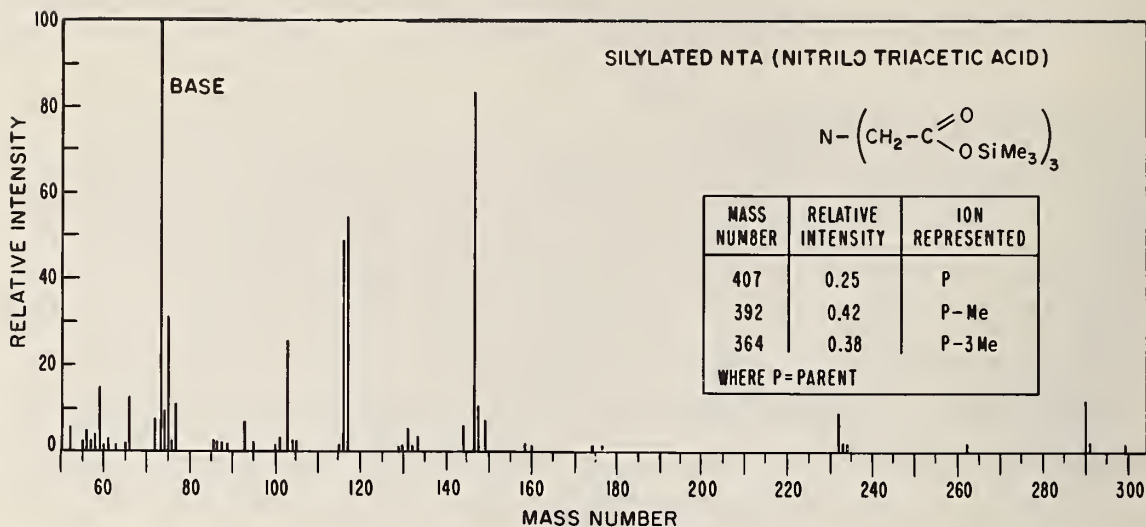
49

Figure 29. Mass spectrum of silylated NTA eluted
            from a gas chromatographic column.

    3. <u>Tetramethylsilane (TMS)</u>. This compound is widely
used as an internal standard for nuclear magnetic resonance
studies. In determining a procedure for trace NMR analysis,
the Organic Chemistry Section at NBS recognized evidence for
contamination. This was then verified in detail by gas
chromatography with the finding of two contaminant peaks at
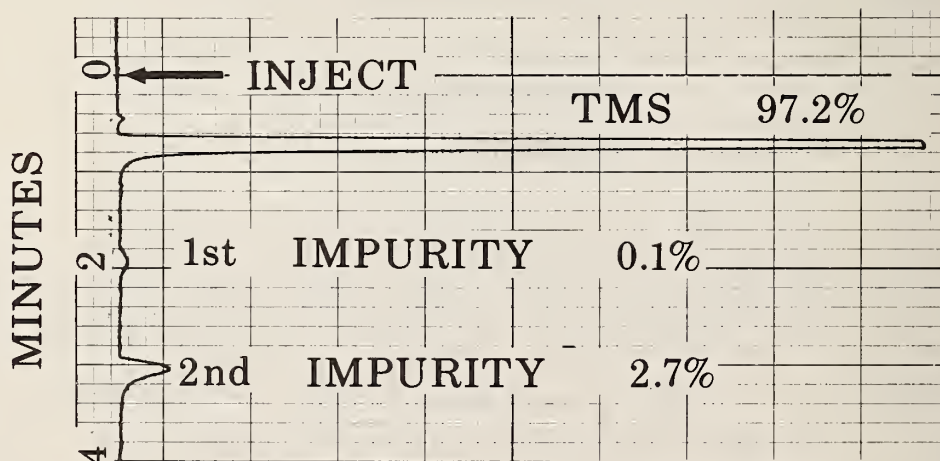2.7 and 0.1 percent. The gas chromatogram is shown in Figure 30.

Figure 30. Gas chromatogram of tetramethylsilane.

3. PERSONNEL AND ACTIVITIES

A. Personnel Listing

Separation and Purification Section

David H. Freeman, Section Chief
Janice M. Hurst, Secretary

Chromatography

Delmo Enagonio
Walter Zielinski
Herbert Dixon
Patti Byrne (summer student)

Ion Exchange Microstandards

Edwin Kuehner

Mass Spectrometry

William Dorko

Network Structures

Rosalie Angeles

Particle Metrology

Herbert Dixon
Robert Young

Ultra-Pure Reagents*

Edwin C. Kuehner

Youth Opportunity Corps

Jennifer Jones

---

*Transferred to Section 310.06

B. Talks

1. R. M. Angeles, "Decomposition and Dehydration of Various Polyelectrolytes Derived from Polystyrene," Sixth Middle Atlantic Regional Meeting, Baltimore, Maryland. February 5, 1971.

2. David H. Freeman, "Donor-Acceptor Liquid Gel Chromatography," State University of New York at Binghamton, Binghamton, New York. February 5, 1971.

3. David H. Freeman, "Acceptor Donor Chromatography," Naval Ordnance Laboratory, Silver Spring, Maryland. February 10, 1971.

4. David H. Freeman, "Hydrogen Bond Liquid Chromatography," Pittsburgh Conference on Analytical Chemistry and Applied Spectroscopy, Inc., Cleveland, Ohio. March 2, 1971.

5. David H. Freeman, "Interactive Gel Chromatography: A Breakthrough in Organic Analysis," University of Maryland, College Park, Maryland. March 23, 1971.

6. David H. Freeman, "Introduction to Liquid Chromatography (Experimental, Theory, Column Selection)," Washington Chromatography Discussion Group, Washngton, D. C. April 2, 1971.

7. David H. Freeman, "High Resolution Liquid-Gel Chromatography University of Florida, Gainesville, Florida. April 16, 1971

C. Publications

1. G. A. Sleater and D. H. Freeman, "Rapid Desorption of Chromium(III) from Cation Exchanger with Hydrogen Peroxide Solutions," Anal. Chem. 42, No. 13, p. 1666-68 (1970).

2. G. A. Sleater, D. H. Freeman, D. H. Olson, and H. S. Sherry, "Zerolite Microstandards," (accepted for publication in Analytical Chemistry).

3. D. H. Freeman and W. L. Zielinski, Jr., "Separation and Purification Section: Summary of Activities July 1969 to June 1970," NBS Technical Note 549 (1971).

4. D. H. Freeman and D. P. Enagonio, "Interactive Gel Networks for Organic Separations," Nature, Physical Science, Vol. 230 135-36 (1971).

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15. SUPPLEMENTARY NOTES

16. ABSTRACT (A 200-word or less factual summary of most significant information. If document includes a significant bibliography or literature survey, mention it here.)

This is the annual progress report on the activities of the Separation and Purification Section. During the past year our studies in liquid chromatography, its application, control, and systems design was continued at an accelerated rate. Mobile phase flow was treated as an analogy to current in direct current circuitry, following removal of liquid pulses generated by a piston pump. A sophisticated, yet simple and inexpensive liquid chromatograph design is fully described. This design has been incorporated into all liquid chromatography studies being conducted in this Section. The operating parameters of this system design are discussed. The development of the underlying theory and synthesis procedures for interactive gels has led to the control and prediction of solute retention, selectivity, and a magnified column capacity for use in fraction collection and ancillary characterization o solute impurities in clinical Standard Reference Materials. A gas chromatographic method is described for the analysis of nitrilotriacetic acid based upon the formation of its trisilyl ester derivative.

17. KEY WORDS (Alphabetical order, separated by semicolons) Clinical Standard Reference Material fraction collecting; gas chromatography; interactive gels; liquid chromatography design; nitrilotriacetic acid.

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